JOURNAL OF CLINICAL PATHOLOGY

EDITED FOR

THE ASSOCIATION OF CLINICAL PATHOLOGISTS

BY

A. GORDON SIGNY

EDITORIAL BOARD

E. N. ALLOTT
J. V. DACIE
J. G. GREENFIELD
R. J. V. PULVERTAFT
DOROTHY S. RUSSELL
JOAN TAYLOR

And the Editor of the *British Medical Journal*

VOLUME V, 1952

LONDON

BRITISH MEDICAL ASSOCIATION

TAVISTOCK SQUARE, W.C.1

CONTENTS

PAGE

No. 1. *FEBRUARY*, 1952

The Mechanism and Interrelationships of the Flocculation Tests. N. F. MACLAGAN, N. H. MARTIN, and J. BARBARA LUNNON 1
The Influence of Age upon Serum Iron in Normal Subjects. ROBERT PIRRIE 10
Amino-aciduria and Copper Metabolism in Hepatolenticular Degeneration. J. D. SPILLANE, J. W. KEYSER, and R. A. PARKER 16
The Nitroprusside Method for the Determination of Blood Glutathione. R. H. S. THOMPSON and D. WATSON 25
The Determination of Blood Glutathione by Amperometric Titration. D. S. BIDMEAD and D. WATSON 30
Necrotizing Pulmonary Arteriopathy Associated with Pulmonary Hypertension. W. ST. C. SYMMERS 36
Liver Glycogen in Diabetes Mellitus. J. VALLANCE-OWEN 42
Primary Amyloidosis with Renal and Myocardial Failure. A. WYNN WILLIAMS 54
Bone-marrow Embolism. R. B. H. TIERNEY 63
Culture of Human Bone Marrow *in vitro*. L. G. LAJTHA 67
The Bone Marrow in Paget's Disease (Osteitis Deformans) with Special Emphasis on the Megakaryocytes. PHILIP PIZZOLATO and LOUIS RAIDER 86
The Effects of a Phenothiazine Derivative (RP. 3300) on Red Cell Preservation. H. CHAPLIN, JUN., HAL CRAWFORD, MARIE CUTBUSH, and P. L. MOLLISON 91
A Comparative Study of Direct Matching Techniques in Blood Transfusion. O. G. DODGE 102
Technical Methods:
A New Type of Blood Film for Differential Counts. MICHAEL F. A. WOODRUFF 108
A New Counting Chamber for the Eosinophil Count. T. MANNERS 111
Combined Sternal Biopsy and Aspiration Needle. B. A. THOMPSON 112
Book Reviews 113
Abstracts 119

No. 2. *MAY*, 1952

The Vitamin B_{12} Concentrations of Serum and Urine of Normals and of Patients with Megaloblastic Anaemias and Other Diseases. D. L. MOLLIN and G. I. M. ROSS 129
Studies on the Pathogenesis of Thalassaemia. GIOVANNI ASTALDI and PAOLO TOLENTINO 140
The Bone Marrow in the Anaemia of Pregnancy. JEAN M. SCOTT and A. D. T. GOVAN 145
The Diagnosis of Carriers of Cooley's Anaemia. FRANCIS S. MOONEY 154
Starch-fermenting, Gelatin-liquefying Corynebacteria and their Differentiation from *C. diphtheriae gravis*. G. T. COOK and W. H. H. JEBB 161
The Antibiotic Sensitivity of Pathogenic Staphylococci. W. D. LINSELL 165
The Incidence of Antibiotic Resistance in Gram-negative Bacilli in a General Hospital. EDGAR F. THOMSON 169
Coronary Arteriosclerosis and Calcification in Infancy. R. A. SLADDEN 175
The Diagnosis of Reticulosarcoma of the Thyroid Gland. G. B. D. SCOTT 183
Serum Copper and its Relationship to Serum Iron in Patients with Neoplastic Disease. ROBERT PIRRIE .. 190
Blood Serum Protein-bound Carbohydrate after Injury. J. W. KEYSER 194
Intrathecal Streptomycin and Cerebrospinal Glucose Estimation. M. J. H. SMITH 199
The Cholesterol Tolerance Test in Normal Individuals. NAZEK I. FAHMY and PAUL GHALIOUNGUI 203
Technical Methods:
A Micro-method for Blood Salicylate Estimations. D. G. MOSS 208
Orthotolidine Hydrochloride Test for Blood in Urine. H. ZWARENSTEIN 212
Association of Clinical Pathologists: 48th General Meeting 213
Abstracts 221

No. 3. *AUGUST*, 1952

PAGE

Primary Epithelial Tumours of the Jaw. T. F. HEWER 225
Lower Nephron Nephrosis after Transurethral Resection of the Prostate. JOHN SWINNEY and B. E. TOMLINSON 234
Anisotropic Non-silicious Crystalline Inclusions in a Dysgerminoma of Tuberculoid Appearance. R. ERIC POTTS 239
The Histological Changes in Hydantoinate Gingivitis. L. CROME 244
The Value of Rib Biopsy in the Study of Marrow Disorders. M. S. R. HUTT, P. SMITH, A. E. CLARK, and J. L. PINNIGER 246
Vitamin B_{12} Assay in Body Fluids using *Euglena gracilis*. G. I. M. ROSS 250
The Construction and Clinical Significance of the Coagulohepatogram. W. RIEBEN 257
The Relation Between Polyagglutinability of Erythrocytes *in vivo* and the Hübener-Thomsen-Friedenreich Phenomenon. J. REEPMAKER 266
Determination of the Unsaturated Iron-binding Capacity of Serum. S. VENTURA 271
The Application of a Haemagglutination Technique to the Study of *Trichomonas vaginalis* Infections. M. G. MCENTEGART 275
The Relationship Between Iron Storage and Anaemia. W. M. DAVIDSON and R. F. JENNISON 281
The Effect of Photo-electric Cell Temperature on Flame Photometer Determinations. A. O. WILSON 286
Technical Methods:
A Rapid Method for Determining Faecal Fat. O. E. NEUFELD 288
A Simple Method for the Aseptic Filling of Screw-capped Containers. J. E. O'HAGAN 290
A Direct Scanning Apparatus for Reading Electrophoretic Paper Strips. L. L. GRIFFITHS 294
Book Reviews 296
Abstracts 298

No. 4. *NOVEMBER*, 1952

Running of Standards in Clinical Chemistry and the Use of the Control Chart. RICHARD J. HENRY and MILTON SEGALOVE 305
The Micro Estimation of Serum Iron and Iron-binding Capacity in Normals and in Disease. G. DAVIES, B. LEVIN, and V. G. OBERHOLZER 312
True Uric Acid Values. J. MAXWELL JOHNSTONE 317
The Carbohydrates in Normal and Abnormal Cerebrospinal Fluid. MARJORIE D. EASTHAM and KENNETH R. KEAY 319
Ox Cell Haemolysins in Human Serum. G. B. LEYTON 324
Extensive Myeloid Response During Folic Acid Therapy in Megaloblastic Anaemia of Pregnancy. GRACE M. RITCHIE 329
Studies on the Normal Serum Panagglutinin Active Against Trypsinated Human Erythrocytes. THEODORE H. SPAET and BETTY W. OSTROM 332
A Case of the Schönlein-Henoch Syndrome with Myocardial Necrosis. M. A. LECUTIER 336
The Histogenesis of Four Cases of Angiomyxoma of the Auricle. C. RAEBURN 339
Polyethylene Glycols as Embedding Media in Histochemical Work. S. SRIRAMACHARI and V. RAMALINGASWAMI 345
Standardization of the Dye Test for Toxoplasmosis. J. K. A. BEVERLEY and C. P. BEATTIE 350
Two Small Colony Variants of *Staph. aureus* Isolated in Pure Culture from Closed Infected Lesions and their Carbon Dioxide Requirements. J. C. SHERRIS 354
Colouring Agents for Use in Disc-antibiotic Sensitivity Tests. J. H. BOWIE and J. C. GOULD 356
Transfer of Fluorescin from *Ps. pyocyanea* to Colonies of Other Bacteria. L. HURST and E. J. L. LOWBURY 359
Media for the Isolation and Differentiation of Pathogenic *Bact. coli* (Serotypes O 111 and O 55). F. RAPPAPORT and E. HENIG 361
The Difficulty Caused by the Fragility of the Ova of *Ascaris lumbricoides*. K. B. ROGERS 363
Scholarships in Aid of Scientific Research 364
Technical Methods:
Device for Sampling Blood Bottles. B. A. THOMPSON 365
The Use of Dextran Sulphate as a Blood Anticoagulant in Biological Research. M. HALL and C. R. RICKETTS 366
Association of Clinical Pathologists: 49th General Meeting 367
Abstracts 373
Index for Volume 5 375

J. clin. Path. (1952), **5**, 1.

THE MECHANISM AND INTERRELATIONSHIPS OF THE FLOCCULATION TESTS

BY

N. F. MACLAGAN, N. H. MARTIN, AND J. BARBARA LUNNON

From the Departments of Chemical Pathology at Westminster Medical School and at St. George's Hospital Medical School, London

(RECEIVED FOR PUBLICATION OCTOBER 23, 1951)

So many flocculation tests have been developed during the last few years that the time appears ripe to attempt a critical review of the mechanism and usefulness of some of the more popular procedures. Although many of these tests were developed primarily in relation to liver disease, where they are of proved value, it is generally recognized that they are not all specific in this respect. There is no doubt, however, that they can throw useful light on the qualitative and quantitative changes in the pattern of the circulating proteins in disease. Earlier work in this field was recently reviewed by one of us (Maclagan, 1948) and the present paper deals with more recent contributions.

Mechanism of the Tests

The mechanism of the tests can best be discussed in relation to the factors known to affect protein solubility. These will be discussed under the following headings: H ion concentration (*p*H) and salt concentration and ionic pattern (of the environment); protein concentration; modification of polar or non-polar protein groups.

Table I compares six commonly used flocculation tests with regard to these factors.

It will be recalled that Hardy in 1905 pointed out that the globulins in particular tended to be insoluble near the acid side of their iso-electric point. If we accept Kekwick's (1940) figure of 6.58 as the iso-electric point of γ-globulin it will be seen that Popper's flocculation test using ammonium sulphate is particularly adjusted to precipitate γ-globulin. In view of the high salt concentration employed (2.0 M) this test may probably be regarded as a modification of the ordinary salting out procedures.

In contrast to this the molarity of the zinc sulphate test is pitched so low (0.001 M) that, although, as Kunkel (1947) has shown so conclusively, its readings are closely correlated with changes in γ-globulin, it can hardly depend upon salting out effects. It seemed probable that in this test an insoluble metal complex is formed, and to test this hypothesis we have carried out the following experiment.

In the first tube a 1% solution of 95% pure γ-globulin prepared by ether fractionation was added to equal volumes of 0.001 molar $ZnSO_4$ solution and distilled water; at the same time in a second tube a 1% solution of the same γ-globulin was added to equal volumes of the same $ZnSO_4$ solution to which had been added an equal volume of a

TABLE I
COMPARISON OF SIX FLOCCULATION TESTS

	*p*H	Ionic Pattern		Approximate Molarity	Protein Concentration (g./100 ml.)
		Cations	Ions		
Ammonium sulphate test (de la Huerga and Popper, 1950)	5·3	NH_4 Na	SO_4 Cl	2·0	0·14
Takata-Ara test (Jezler, 1930)	12·0	Hg Na	Cl (CO_3)	0·1	3·0–0·3
Cadmium sulphate test (Wuhrmann and Wunderly, 1947)	7·0	Cd	SO_4	0·1	4·6
Thymol test (Maclagan, 1944b)	7·8	Thymol Na	Barbitone	0·01	0·14
Zinc sulphate test (Kunkel, 1947)	7·5	Zn Na	SO_4 Barbitone	0·001	0·14
Colloidal gold test (Maclagan, 1944a)	7·8	Au Na Phenol	Barbitone Cl Citrate	0·01	0·14

saturated solution of pure glycine in distilled water. As a control to the two systems a third tube of the original 1% γ-globulin solution was diluted with two volumes of distilled water. The three systems were examined periodically over 48 hours at 20° C.

Flocculation occurred in the first tube within five minutes followed by precipitation in two hours, and at the end of two hours the third, control, tube was slightly opalescent, the second tube containing the glycine remaining clear and unchanged over the whole period of the observation. The glycine has a stability constant with Zn of 9.2 as measured in the laboratory where this test was carried out (Perkins, 1952) which compares closely with the published figure of 9.3 of Albert (1950). Zn has a higher co-ordinating capacity than either ferric or ferrous iron, iron being the only metal likely to be present in the system capable of forming co-ordinating complexes.

Our interpretation of this simple experiment is that at the concentration described the glycine forms a soluble co-ordination complex with the Zn ions available which are not therefore available to co-ordinate with the γ-globulin, the stability constant of which is probably much lower than glycine.

While the only unassailable evidence would be the isolation and characterization of the zinc globulin complex we do believe that this experiment is extremely suggestive, especially when taken in conjunction with Lewin's (1951) work on the crystallization of the metallic salts of albumin.

The colloidal gold test presumably depends upon a similar mechanism as it operates at a similar *p*H and ionic strength (0.01 M). It will be shown later that the gold and zinc sulphate tests are strongly correlated with each other.

The mechanism of the thymol test appears to be more complex. Unlike the other tests considered there is little correlation with total serum globulin (see below). Maclagan and Bunn (1947) demonstrated that in this test turbidity only occurs in the presence of phospholipid. This would normally be contributed by the β-globulin fraction, but the addition of cephalin was an effective substitute. (This may explain the observations of Cohen and Thompson (1947) that the β-globulin which is rich

in phospholipid is partially carried down in the thymol precipitate.) In the presence of cephalin γ-globulin was the only fraction that produced turbidity.

Maclagan and Bunn also showed that albumin inhibited the reaction and that hepatitis albumin was a less effective inhibitor than normal albumin. Martin (1948, 1949), studying the inhibitory effect of crystalline human and bovine albumin on the thymol reaction, confirmed these findings and stressed particularly the differences between normal and hepatitis albumin. These qualitative changes in the albumin fraction are evidently of the greatest significance in this test. Martin also described an unusual type of γ globulin showing spontaneous precipitation during electrophoresis and giving positive flocculation tests when added to normal serum.

TABLE II

EFFECT OF INTRAVENOUS ALBUMIN ADMINISTRATION ON FIVE PATIENTS WITH POSITIVE FLOCCULATION TESTS

Albumin (g. intravenously)	50	58	60	75	100
Thymol test	55%*	60%	50%	50%	65%
Zinc sulphate test	20%	33%	25%	—	25%
$(NH_4)_2SO_4$ test	15%	30%	20%	—	15%
Colloidal gold test	12%	20%	—	—	—

* % reduction of flocculation in units.

It remained to study the effect of albumin administered *in vivo*. Table II shows the results of five such experiments and indicates the important part that normal albumin plays in preventing abnormal thymol flocculation, for in every instance the intravenous administration of albumin produced a fall in the intensity of the thymol reaction. The other flocculation tests were similarly affected but to a smaller extent. Even more striking is a serial study carried out on a patient with extensive liver necrosis who received a single transfusion of serum albumin (Fig. 1).

The base line in Fig. 1, 100%, represents the levels estimated at three-day intervals over seven days before the intravenous administration of 60 g. of albumin dissolved in 300 ml. of 0.15 molar saline. Subsequent levels were estimated at the completion of the transfusion, 18 hours after and 48 hours after, and thereafter at the intervals shown on the chart. Fig. 1 shows that, 20 days after transfusion, the flocculation tests were all within the limits of the base line, and that in the interval between the levels were reduced to a varying extent ; the ammonium sulphate test least, the zinc sulphate test next, and the thymol test to the greatest extent for the greatest period of time. It is noteworthy that the depression of the thymol test corresponded roughly to the expected time of survival of a significant amount of the infused albumin.

The complexity of factors involved in the thymol test will be recognized from the above discussion, but it is evident that qualitative and quantitative changes in the albumin and γ-globulin fractions are the most important items.

Less work appears to have been done on the mechanism of the cadmium sulphate and Takata-Ara tests. These both employ an intermediate ionic strength (0.1 M) together with metallic cations and perhaps depend upon a combination of salting out with the formation of insoluble metal complexes. The very high *p*H of the

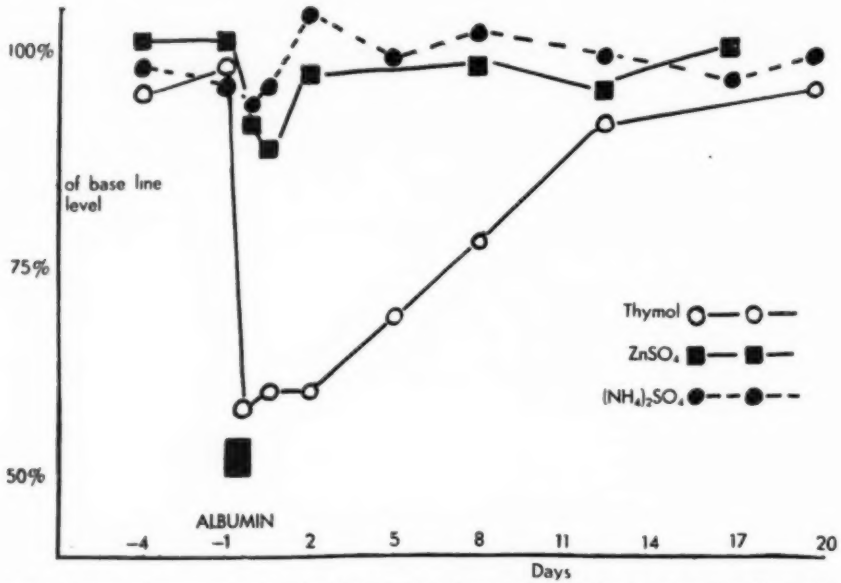


FIG. 1.—Effect of intravenous albumin administration (60 g.) on flocculation tests in a case of hepatitis.

Takata-Ara test is noteworthy (about 12.0) and must produce some degree of denaturation of the proteins concerned.

Correlation Between Tests

It is well recognized that there is a strong family resemblance between the various tests and a pronounced tendency to correlation between them, while at the same time considerable discrepancies may occur in individual cases. To obtain further information on this point we have examined the correlation coefficients between the five tests in which we have been mainly interested, taken in pairs. The correlation

TABLE III

CASES USED FOR CALCULATION OF CORRELATION COEFFICIENTS

Test	Diagnosis			
	Hepatitis	Obstructive Jaundice	Miscellaneous*	Totals
Thymol	93	30	117	240
Gold	93	30	117	240
$ZnSO_4$	93	30	117	240
$(NH_4)_2SO_4$	53	13	52	118
Serum proteins ..	23	5	55	83

* "Miscellaneous" includes all cases other than hepatitis and obstructive jaundice, omitting those with all tests normal.

of each test with total serum globulin and with the albumin/globulin ratio was also examined.

The material used in this study is shown in Table III and includes all cases seen under the relevant headings with certain exceptions. Thus 298 miscellaneous cases (i.e. cases other than hepatitis or obstructive jaundice) in which every test was negative are excluded, and in addition 52 cases in which the diagnosis was uncertain and 14 cases of multiple myelomatosis were excluded. The last was necessary since it had previously been shown that there was a special tendency to dissociation between the tests in this condition (Earle, Martin, and Maclagan, 1951). This point will be further discussed in the next section.

The following list gives the methods used :

Test	Reference	Normal Limits
Thymol turbidity test	Maclagan (1944b)	0–2 units
Thymol flocculation test	Maclagan (1947)	0–1+
Serum colloidal gold reaction	Maclagan (1944a)	0
Zinc sulphate turbidity test	Kunkel (1947)	0–4 units
Ammonium sulphate turbidity test ..	de la Huerga and Popper (1950)	0–2 ,,

The turbidity units for all tests are based on diluted normal serum treated with salicylsulphonic acid as described by Maclagan (1947). They are therefore not identical with the units proposed by the authors of the zinc sulphate and ammonium sulphate turbidity tests. The normal limits were those obtained with a group of 30 normal subjects.

The serum protein estimations were made by the micro-Kjeldahl method, using a Markham (1942) all-glass distillation apparatus. The globulin was precipitated with

TABLE IV

CORRELATION COEFFICIENTS (± STANDARD ERRORS) BETWEEN FLOCCULATION TESTS AND SERUM PROTEINS IN NON-MYELOMA CASES

	Thymol Flocculation	Thymol Turbidity	Serum Colloidal Gold	Zinc Sulphate Turbidity	Ammonium Sulphate Turbidity	Total Serum Globulin	A/G Ratio
Thymol flocculation	1·0	0·78 ±0·025	0·68 ±0·035	0·64 ±0·38	0·39 ±0·078	0·15 ±0·107	0·07 ±0·109
Thymol turbidity	0·78 ±0·025	1·0	0·60 ±0·041	0·63 ±0·039	0·50 ±0·069	0·23 ±0·104	0·13 ±0·108
Serum colloidal gold	0·68 ±0·035	0·60 ±0 041	1·0	0·73 ±0 030	0·45 ±0·073	0·40 ±0·092	0·29 ±0·101
Zinc sulphate turbidity ..	0·64 ±0·038	0·63 ±0·039	0·73 ±0·030	1·0	0·76 ±0 053	0·60 ±0·070	0·47 ±0·088
Ammonium sulphate turbidity	0·39 ±0·078	0·50 ±0·069	0·45 ±0·073	0·76 ±0·053	1·0	0·65 ±0·089	0·42 ±0·127
Total serum globulin	0·15 ±0·107	0·23 ±0·104	0·40 ±0·092	0·60 ±0·070	0·65 ±0·089	1·0	0·83 ±0 034
A/G ratio	0·07 ±0·109	0·13 ±0·108	0·29 ±0·101	0·47 ±0·088	0·42 ±0·127	0·83 ±0·034	1·0

The signs of the correlation coefficients are omitted from the table. They were all positive except for the A/G ratio figures, which were negative.

22% w/v Na_2SO_4. The proteins were digested for two hours after clearing, using a selenate catalyst.

The results of this work are shown in Table IV, two typical correlation diagrams being illustrated in Figs. 2 and 3. Owing to the fact that different numbers of cases have been employed in some correlations as compared with others, the standard errors will be seen to vary somewhat, but as a rough guide it may be taken that any pair of correlation coefficients differing by more than 0.1 are probably statistically different. For example, 0.64 (zinc sulphate turbidity and thymol flocculation) in the table is significantly different from 0.78 (thymol turbidity and thymol flocculation) but does not differ significantly from 0.68 (gold and thymol flocculation).

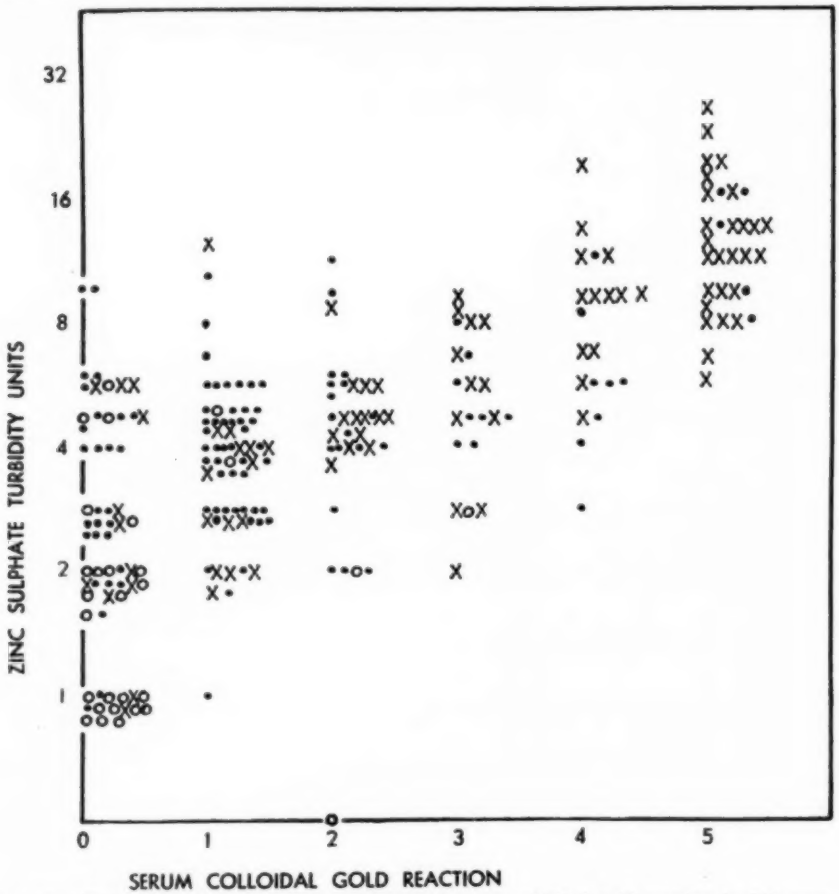


FIG. 2.—Correlation between $ZnSO_4$ turbidity and serum colloidal gold reaction in 240 cases. X = hepatitis; O = obstructive jaundice; ● = miscellaneous. Correlation coefficient $0{\cdot}73 \pm 0{\cdot}030$.

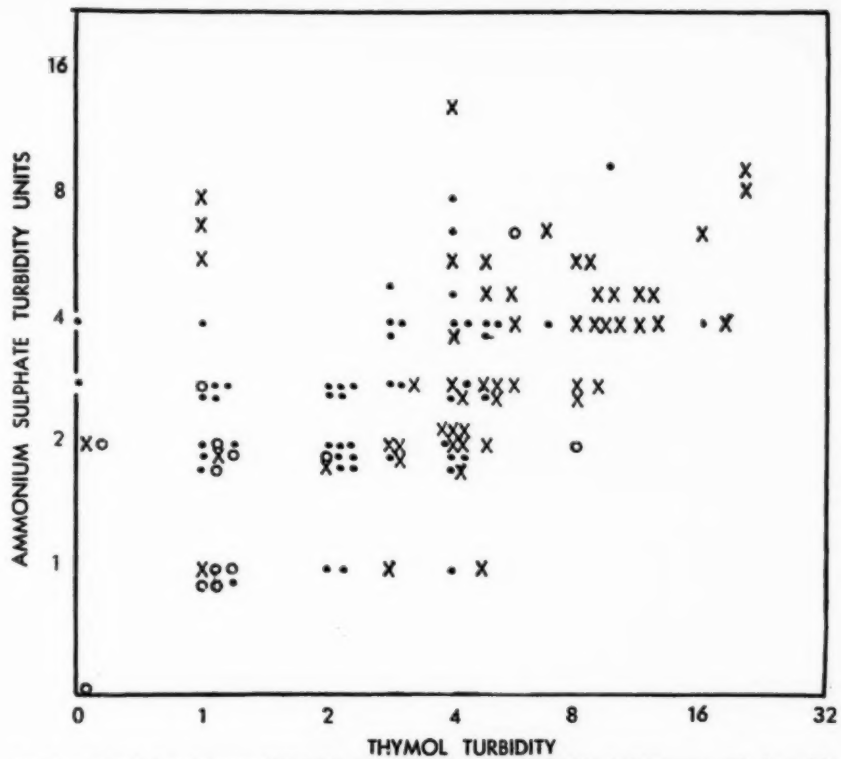


FIG. 3.—Correlation between $(NH_4)_2SO_4$ turbidity and thymol turbidity in 118 cases. X = hepatitis; O = obstructive jaundice; ● = miscellaneous. Correlation coefficient $0{\cdot}50 \pm 0{\cdot}069$.

It will be seen from Table IV that it has been possible to arrange the tests in the following order : albumin/globulin ratio, total serum globulin, ammonium sulphate turbidity, zinc sulphate turbidity, serum colloidal gold reaction, thymol turbidity and flocculation, which expresses the tendency of the tests to correlate with one another. Thus the tests appearing close together in this list are more strongly correlated than those which are more widely separated. This is illustrated by the gradation of correlation coefficients as one proceeds across or down the table.

It is also evident that there is a tendency for the tests to be arranged in three groups : (1) thymol turbidity and flocculation ; (2) gold and zinc sulphate ; and (3) ammonium sulphate and total serum globulin. The correlation within each group is much closer than that between the groups, and group 2 occupies an intermediate position between groups 1 and 3 as shown by the higher correlation between groups 1 and 2 and between groups 2 and 3 as opposed to the lower correlation between groups 1 and 3. The albumin/globulin ratio occupies a position rather outside these three groups as it shows a weak correlation with a few of the tests only. The lower

correlation between the thymol tests and the albumin/globulin ratio as compared with the serum globulin is somewhat surprising. Although it is not statistically significant for each individual test, this general trend appears to be quite clear. The explanation may probably lie in the variation in the extent of the qualitative changes in the albumin from one serum to another. These qualitative changes are not of necessity mirrored in the albumin/globulin ratio.

It is interesting that the order of tests obtained by this study of correlation is almost identical with the order indicating the probability of obtaining positive results in myelomatosis (see below). The relationships outlined here are not unexpected in view of the different mechanisms of the tests referred to above. Thus, the ammonium sulphate test and the total globulin estimation are operating in high salt concentration, the gold and zinc sulphate tests probably depend upon some specific interaction with metallic cations at low ionic strength, whereas the thymol test evidently depends upon some entirely different mechanism.

Myelomatosis

Whereas in a case of hepatitis most of the different flocculation tests are usually either positive or negative, in a case of myelomatosis it is frequently found that some tests are strongly positive while others are completely negative (Earle, Martin, and Maclagan, 1951).

We have collected some further evidence on this point and the results of the various tests in our total series of cases of plasmacytoma are shown in Table V. It will be seen that this confirms our previous finding of the marked tendency to dissociation between the tests in this disease, a dissociation which exceeds that seen in any other condition. It was also found that the tests could be arranged in a definite order corresponding to the probability of obtaining positive results. This is the order shown in the table and is total serum globulin, albumin/globulin ratio,

TABLE V

FLOCCULATION TESTS AND SERUM PROTEINS IN 14 CASES OF PLASMACYTOMA

Case No.	Total Serum Globulin (g.%)	A/G Ratio	$(NH_4)_2SO_4$ Turbidity	$ZnSO_4$ Turbidity	Serum Colloidal Gold Test	Thymol Turbidity	Thymol Flocculation
1	3·1	*1·61**		*3*	*0*	*1*	*0*
2	*1·4*	*3·50*		*1*	*0*	*1*	*0*
3	8·5	0·86		5	1+	*1*	*0*
4	6·4	0·42		*0*	*0*	*0*	*0*
5	5·8	0·57	*2*	*0*	*0*	*0*	*0*
6	7·0	0·34	+++†	*0*	*0*	*0*	*0*
7	6·0	0·55	4	*0*	*0*	*0*	*0*
8	10·2	0·24	*2*	*2*	*0*	*1*	*0*
9	9·4	0·21	7	18	*0*	*1*	*0*
10	8·0	0·05	32	80	5+	12	4+
11	8·3	0·22	21	*0*	*0*	*2*	*0*
12	10·1	0·24	21	*0*	*0*	*1*	*0*
13	5·7	0·72	21	28	*0*	*1*	*0*
14	6·7	0·43	18	28	5+	*0*	*0*

* Normal results in italics.

† Immediate heavy flocculation, turbidity could not be measured.

ammonium sulphate turbidity, zinc sulphate turbidity, serum colloidal gold reaction, thymol turbidity, and flocculation. The tests at the beginning of this list are more likely to be positive than those at the end, and in our small series we have so far encountered no case of myeloma showing the converse of this. If, for example, the zinc sulphate test is negative the gold and thymol are negative, whereas if the thymol test is positive all the other tests are positive. It is interesting that this order of tests is the same as that given by the study of correlation coefficients in non-myeloma cases reported above, with the exception of the total globulin and albumin/globulin ratio which are in the reverse order in the two lists.

The most frequent finding in myelomatosis appears to be a high globulin with low albumin/globulin ratio and strongly positive ammonium sulphate and zinc sulphate, the remaining tests being negative. It will be seen from Table IV that a degree of dissociation sufficiently striking for diagnostic purposes was found in 12 out of 14 cases, the two exceptions being Case 2 (all results normal) and Case 10 (all results abnormal). In our opinion this type of dissociation is a valuable diagnostic pointer in myelomatosis as we have not seen it in any other disease.

Summary

The fundamental factors contributing to a number of flocculation tests have been reviewed and the degree of correlation of one test with another has been examined. Evidence is produced to show that on this basis the flocculation tests which we have studied may be grouped into three main families. The tests within each group are closely correlated, but the correlation between groups is less close.

(1) Tests, such as the ammonium sulphate, which may be regarded primarily as modifications of the classical salting out procedures; (2) the colloidal gold and zinc sulphate tests in which the formation of a metallic complex is the probable fundamental mechanism; (3) tests, such as the thymol test, in which complex formation of a more complicated nature is concerned, probably involving a protein-protein linkage.

The advantage of using a battery of tests is stressed, particularly in the case of myelomatosis in which there is a special and perhaps unique tendency to dissociation between the tests.

REFERENCES

Albert, A. (1950). *Biochem. J.*, **47**, 531.
Cohen, P. C., and Thompson, F. (1947). *J. Lab. clin. Med.*, **32**, 314.
Earle, J. H. O., Martin, N. H., and Maclagan, N. F. (1951). Proc. Ass. clin. Path., *Journal of Clinical Pathology*, **4**, 117.
Huerga, J. de la, and Popper, H. (1950). *J. Lab. clin. Med.*, **35**, 459.
Hardy, W. B. (1905). *J. Physiol., Lond.*, **33**, 251.
Jezler, A. (1930). *Z. klin. Med.*, **114**, 739.
Kekwick, R. A. (1940). *Biochem. J.*, **34**, 1248.
Kunkel, H. G. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 217.
Lewin, J. (1951). *J. Amer. chem. Soc.*, **73**, 3906.
Maclagan, N. F. (1944a). *Brit. J. exp. Path.*, **25**, 15.
—— (1944b). Ibid., **25**, 234.
—— (1947). *Brit. med. J.*, **2**, 197.
—— (1948). Ibid., **2**, 892.
—— and Bunn, D. (1947). *Biochem. J.*, **41**, 580.
Markham, R. (1942). Ibid., **36**, 790.
Martin, N. H. (1948). *Nature, Lond.*, **162**, 145.
—— (1949). *Journal of Clinical Pathology*, **2**, 275.
Perkins, D. J. (1952). In the press.
Wuhrmann, F., and Wunderly, C. (1947). *Die Bluteiweisskörper des Menschen*. Basel.

J. clin. Path. (1952), **5**, 10.

THE INFLUENCE OF AGE UPON SERUM IRON IN NORMAL SUBJECTS

BY

ROBERT PIRRIE

From the Muirhead Department of Medicine, the University and Royal Infirmary, Glasgow

(RECEIVED FOR PUBLICATION MAY 1, 1951)

This investigation was carried out in order to explore the possibility of a correlation between the process of ageing and the concentration of iron in the serum. It also provides additional information on the reliability of the potassium ferricyanide method for the determination of serum iron.

Vahlquist (1941) studied the influence of age upon serum iron concentration during the first 15 years of life. Within 24 hours of birth, serum iron falls from its neonatal value of 160 μg. % to a level of 50 μg. %. A secondary rise to 125 μg. % during the next two weeks is succeeded by a gradual fall so that the average value in the age group 6 months to 2 years is 60 μg. %. After the second year the concentration of iron in the serum increases progressively to an average of 100 μg. % at 7 years, 110 μg. % at 11 years, and 120 μg. % at 15 years. Eckerström (1944a, b) determined serum iron in 100 subjects over 70 years of age and found that not only was the sex difference in serum iron still apparent at these ages, but also that serum iron was significantly lower than in healthy subjects under 30 years of age.

Material and Methods

The material was composed mainly of blood donors, namely healthy individuals between the ages of 17 and 65 years of age, who gave no history of recent illness, whose physical condition was good, and whose haemoglobin was 85% or more, as estimated by the copper sulphate method of Philips, Van Slyke, Dole, Emerson, Hamilton, and Archibald (1945). The age groups 10 to 17 years and 65 to 80 years were composed respectively of children with minor injuries and of adult volunteers whose health standard conformed to the above criteria. Females who were menstruating were rejected. All specimens were withdrawn in the afternoon. In the case of blood donors, serum iron was determined on samples removed immediately after withdrawal of 540 ml. of blood. This procedure was desirable, since it minimized interference with the regime of the transfusion service, but was adopted only after it was shown that the iron content of the serum was not influenced significantly by the immediately preceding donation of blood. This observation agrees with that of Heilmeyer and Plötner (1937), who noted that the withdrawal of 500 to 800 ml. of blood from normal persons did not reduce serum iron until at least six hours later. Likewise, Brφchner-Mortensen (1943), who studied two cases of acute gastro-intestinal haemorrhage from the onset, obtained normal values for serum iron during the first 24 hours.

Serum iron was determined by the potassium ferricyanide method of Fitzpatrick and Howells (1949) by whom it has been described in detail. Throughout the estimations every precaution was taken to ensure freedom of contamination with iron. The final colour was measured by a Spekker absorptiometer, for which a calibration curve was constructed by reference to serial dilutions of a standard solution of ferrous ammonium sulphate.

Results

As a measure of the experimental error involved, the iron content of aliquot portions of the same serum was estimated in duplicate in ten different individuals chosen at random (Table I). The difference in the mean values of these determinations is only 0.2 μg. %. The standard error of a single determination is ±5.5 μg. %, so that any single determination of serum iron can be considered liable to a maximum error of ±11 μg. %.

TABLE I

DUPLICATE ESTIMATIONS OF SERUM IRON

Serum	Iron Content (μg. %)		Difference
	1	2	
Serum 1	170	165	−5
,, 2	112	120	+8
,, 3	93	81	−12
,, 4	113	120	+7
,, 5	60	60	0
,, 6	70	80	+10
,, 7	100	93	−7
,, 8	111	119	+8
,, 9	119	119	0
,, 10	140	133	−7

In order to establish that serum iron was not immediately influenced by the withdrawal of 540 ml. of blood, serum iron was determined in six males and six females immediately before and after blood donation. The means and the standard errors of the results are presented in Table II. There is no significant difference between serum iron values obtained before or after donation of blood in either sex.

TABLE II

SERUM IRON ESTIMATED IMMEDIATELY BEFORE AND AFTER WITHDRAWAL OF 540 ML. OF BLOOD

	Mean Values (μg. %)		Difference (μg. %)	Standard Error of Difference of Means (μg. %)
	Before	After		
Males	94·5	98·8	4·3	±3·9
Females	87·3	83·8	3·5	±3·9

The values obtained for serum iron in the various age groups of normal individuals are summarized in Table III and compared with those obtained by previous workers in Table IV. The mean values for serum iron and the standard errors are 103 ± 3.10 μg. % in men and 91 ± 2.83 μg. % in women. These results are lower

TABLE III

MEAN VALUES FOR SERUM IRON IN NORMAL SUBJECTS

Age (Years)	Males			Females		
	Number of Subjects	Range (μg. %)	Mean (μg. %)	Number of Subjects	Range (μg. %)	Mean (μg. %)
10–15	4	65–120	97	5	66–112	92
16–20	5	112–148	129	7	86–137	116
21–30	18	92–188	132	15	74–143	116
31–40	26	76–170	115	12	56–142	100
41–50	19	74–130	96	19	45–137	89
51–60	16	55–130	90	21	35–119	76
61–70	10	55–101	84	12	45–111	75
71–80	10	55–111	75	7	45–95	66
10–80	108	55–188	103	98	35–143	91

TABLE IV

SUMMARY OF PUBLISHED DATA ON SERUM IRON IN NORMAL SUBJECTS

Investigator	Date	Males			Females		
		No.	Mean (μg. %)	Range (μg. %)	No.	Mean (μg. %)	Range (μg. %)
Moore and others	1937	15	121	94–174	15	97	58–142
Heilmeyer and Plötner	1937	25	126	81–162	25	89	64–128
Brøchner-Mortensen and Olsen	1940	20	132	90–194	20	127	79–191
Vahlquist	1941	50	142	68–263	50	123	53–210
Høyer	1944	50	131	63–189	50	114	69–197
Laurell	1947	61	124	70–214	39	108	57–196
Cartwright and Wintrobe	1949	15	127	79–196	15	123	101–164
Rath and Finch	1949	15	106	87–147	15	94	72–130
Tötterman	1949	67	140	88–276	66	121	70–202
Pirrie	1951	108	103	55–188	98	91	35–143
Pirrie (subjects between 16 and 40 years)	1951	49	126	65–188	34	111	56–142

than those previously reported. It is considered, however, that the cause of this discrepancy is the introduction of older age groups in the present series so that its average age is considerably higher than that of previous ones. When all individuals in the age groups 16 to 40 years are selected so as to provide material comparable to that used in previous investigations, mean values of 126 μg. % in 49 males and 111 μg. % in 34 females are obtained. These figures are in excellent agreement with those already published.

The difference between the sexes in this series is 12 ± 4.2 μg. % and is statistically significant. This difference is of the usual magnitude (Table IV). At puberty serum iron increases with age in both sexes, but the rise is greater in males and in this way the sex difference arises. Sex differentiation is maximal during the reproductive period of life and there is no tendency for serum iron to increase after the menopause.

The mean values for serum iron in the various age groups are presented in pictorial form in Fig. 1. Apart from the initial rise between puberty and the age group 20 to 30 years, there is a progressive and statistically highly significant fall in iron concentration in the serum with progressive increase in age. The rate of fall is approximately the same in both sexes.

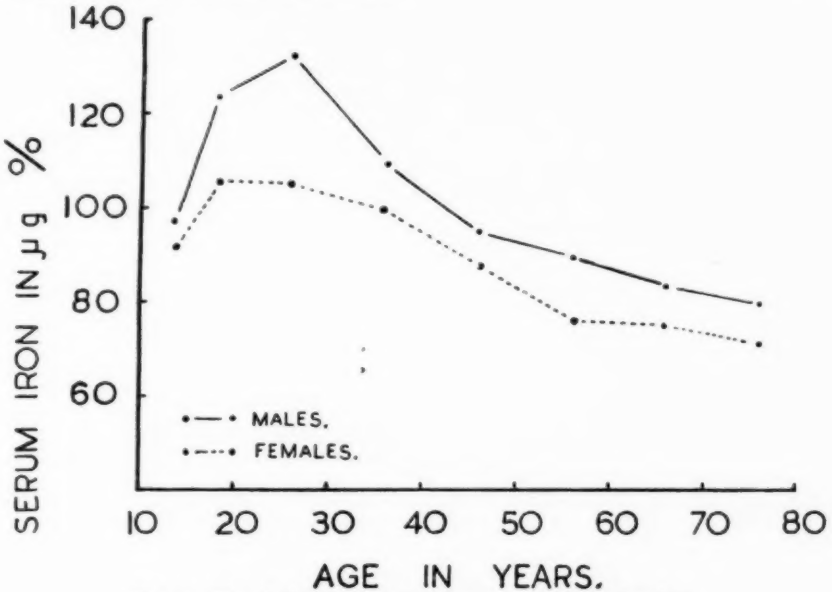


FIG. 1.—The relationship of serum iron to the process of ageing.

Discussion

The use of blood donors as normal material in an investigation of this kind is justified provided the usual precautions of routine examination and adequate interval between donations are observed. Under these circumstances, the possibility of cumulative iron deficiency is negligible. Hynes (1949) estimates that the usual donation of 450 ml. of blood contains 240 mg. and 220 mg. of iron in men and women respectively. Under normal conditions this amount of iron would be replaced by absorption of dietary iron within 60 days in men and 73 days in women. Hynes concludes, therefore, that iron deficiency would not occur, even in normal women, unless blood were donated more than five times per year. The minimum period between donations by subjects included in this series is six months.

Vahlquist's contention (Vahlquist, 1941) that the difference in serum iron between sexes is due to an endocrinal factor and not to iron loss incurred by menstruation is supported by the present study, for not only does the sex difference arise from a proportionally greater increase in serum iron in males at puberty, but also reaches its maximum during the reproductive period of life, i.e. the period during which the activity of endocrinal factors is also at its maximum. During the same period, of course, the demand upon the iron reserves of women is also at its greatest, but cannot be the sole explanation of the maximum difference occurring at this time since there is no corresponding decrease in serum iron.

Little can be said at this stage about the progressive decrease in serum iron with the process of ageing, except to record it as a manifestation of the process. Iron may be less efficiently absorbed by older people, although it is interesting to note in this connexion that Eckerström (1944a) could find no correlation between achlorhydria and low serum iron in his series of aged subjects. Perhaps the general decrease in metabolic activity associated with ageing may be the explanation, in that the demand for the iron containing respiratory enzymes will be correspondingly diminished.

A study of the iron-combining power of the plasma would throw light on this problem. If decreased demand for iron were indeed the explanation of this hypoferraemia of the aged, then a corresponding diminution in plasma-combining power would be expected. On the other hand, if the low serum iron is a manifestation of iron deficiency, more potential than real since haemoglobin values are within normal limits, then the necessity of more efficient absorption and transportation of iron would be reflected by an increase in iron-combining power in the plasma. Laurell (1947) failed to establish a relationship between iron-combining power and ageing in his series of normal subjects, the majority of whom, however, fell between the ages of 15 and 40 years.

Values obtained for serum iron in normal subjects by various workers show a remarkable consistency despite the multitude of techniques employed. The results of this investigation show that the potassium ferricyanide method of Fitzpatrick and Howells (1949) is equally consistent. Its relative simplicity and the stability of the coloured complex make the method additionally attractive, especially when large numbers of determinations have to be carried out.

Summary

Mean values of 103 ± 3.10 μg. % in males and 91 ± 2.83 μg. % in females were established for serum iron in a series of 108 males and 98 females of ages varying from 10 to 80 years. The observed sex difference of 12 ± 4.20 μg. % was maximal during the reproductive period of life.

A progressive and highly significant decrease in serum iron with increasing age was noted and a tentative explanation offered.

The potassium ferricyanide method is a reliable method for the estimation of serum iron.

During this investigation the author held the Faulds Research Fellowship of Glasgow University, and part of the expenses incurred was provided by the Rankin Fund.

I have pleasure in recording my indebtedness to Professor L. J. Davis for his helpful advice, to Dr. J. Wallace for facilitating access to blood donors, and to Dr. R. A. Robb for his help in statistical methods.

References

Brøchner-Mortensen, K. (1943). *Acta med. scand.*, **113**, 345.
—— and Olsen, C. (1940). *Nord. Med.*, **8**, 2502.
Cartwright, G. E., and Wintrobe, M. M. (1949). *J. clin. Invest.*, **28**, 86.
Eckerström, S. (1944a). *Nord. Med.*, **21**, 276.
—— (1944b). Ibid., **24**, 1843.
Fitzpatrick, J., and Howells, K. W. (1949). *Journal of Clinical Pathology*, **2**, 290.
Heilmeyer, L., and Plötner, K. (1937). *Das Serumeisen und die Eisenmangelkrankheit*. Jena.
Høyer, K. (1944). *Acta med. scand.*, **119**, 562.
Hynes, M. (1949). *Journal of Clinical Pathology*, **2**, 99.
Laurell, C. B. (1947). *Acta physiol. scand.*, **14**, Suppl. 46.
Moore, C. V., Arrowsmith, W. R., Quilligan, J. J., and Read, J. T. (1937). *J. clin. Invest.*, **16**, 613.
Philips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., and Archibald, R. M. (1945). *Copper Sulphate Method for Measuring Specific Gravities of Whole Blood and Plasma*. New York.
Rath, C. E., and Finch, C. A. (1949). *J. clin. Invest.*, **28**, 79.
Tötterman, L. E. (1949). *Acta med. scand.*, **134**, Suppl. 230.
Vahlquist, B. (1941). *Acta paediatr., Stockh.*, **28**, Suppl. 5, 1.

J. clin. Path. (1952), **5**, 16.

AMINO-ACIDURIA AND COPPER METABOLISM IN HEPATOLENTICULAR DEGENERATION

BY

J. D. SPILLANE, J. W. KEYSER, AND R. A. PARKER

From the Departments of Neurology and Neurosurgery and of Pathology and Bacteriology, Welsh National School of Medicine, Cardiff

(RECEIVED FOR PUBLICATION JULY 30, 1951)

There is no doubt about the importance of the discovery by Uzman and Denny-Brown (1948) of severe and persistent amino-aciduria in a case of hepatolenticular degeneration. No evidence of renal disease was found in their case, and there was neither clinical nor laboratory evidence of hepatic cirrhosis. It was therefore natural to consider the possibility of there being a fundamental defect in the metabolism of amino acids in this disease. The condition is distinguished from the Fanconi syndrome, which shows amino-aciduria but has other associated metabolic disorders. Uzman and Denny-Brown's patient was suffering from the mildest form of the disorder, and liver biopsy showed that the lobular arrangement was somewhat distorted but that there was no definite increase of the connective tissue in the portal areas. A brother was similarly afflicted, but biochemical studies were not made.

Porter (1949) also found that in three cases of the disease there was consistent amino-aciduria, and that in examples of diseases such as Huntington's chorea, paralysis agitans, dystonia musculorum deformans, and familial spastic paraplegia no such abnormality was present. Eckhardt, Cooper, Faloon, and Davidson (1948), at about the same time as Uzman and Denny-Brown, reported that five cases of Wilson's disease excreted in the urine greater amounts both of α-amino nitrogen and of 10 "essential" amino acids than did eight normal subjects. Seven patients with severe liver disease showed considerable individual variation in amino acid excretion, with an average not much above that of the normals.

The same group of workers (Cooper, Eckhardt, Faloon, and Davidson, 1950) confirmed the presence of amino-aciduria in six cases of hepatolenticular degeneration. They also found that their patients had on the average a slightly higher fasting plasma α-amino nitrogen concentration than did the normals. This was inconstant, however, and no correlation could be demonstrated between the amino-aciduria and the plasma amino nitrogen concentration. All of their patients had some indication of liver disease, although in only one was it severe. Again, there was no correlation between the extent of the amino-aciduria and the severity of the liver disease. They found, by contrast, that other, control, patients with moderate or severe cirrhosis of the liver did not have increased excretion of amino acids in the urine. They inferred that to attribute the amino-aciduria in hepatolenticular degeneration to the hepatic involvement it would be necessary to assume that the variety of liver abnormality is distinctly different from that of ordinary cirrhosis.

It would also be an unusual hepatic defect that resulted in amino-aciduria without elevating the plasma amino nitrogen concentration.

Cooper and his colleagues found that the rapid infusion of amino acids to their patients with hepatolenticular degeneration did not result in a significantly greater loss of amino acids than in normals. There was no demonstrable defect of intermediary amino acid metabolism. The persistence of the amino-aciduria in the fasting state suggested to them that there was a lowered renal threshold for amino acids. In three of their patients there was renal glycosuria, and another patient presented evidence of deranged calcium and phosphorus metabolism and osteomalacia. They concluded that the kidney was probably the site of the defect resulting in excessive amino acid loss, but the type of abnormality remains unexplained. It is possible, however, as suggested by Cooper and his colleagues, that some abnormality in the kidney would lead to a loss of one or more substances necessary for the normal function of brain and liver.

In the muscular dystrophies generalized amino-aciduria has been recorded by Ames and Risley (1948), but it there appears to be a reflection of the severity of the disease and the extent of the muscular wasting.

A second abnormality that may be present in hepatolenticular degeneration consists of an increase in the copper content of both brain and liver (Haurowitz, 1930; Glazebrook, 1945; Cumings, 1948). Glazebrook found in his case that the blood and liver copper were respectively 0.30 mg./100 ml. and 4.6 mg./100 g. (presumably wet tissue). He also considered that the figures for the copper content of cerebral cortex and basal ganglia were elevated. Cumings, in three cases that came to necropsy, found that in each there was an increased copper content in the brain and liver. His figures were (mg./100 g. dry tissue):

Cortical white matter	10.9,	14.7,	12.9
Cortical grey matter	4.6,	27.6,	46.5
Caudate	10.1,	31.8,	13.8
Thalamus	31.9,	20.7,	31.9
Putamen	— ,	60.5,	69.3
Globus pallidus	8.4,	23.0,	39.9
Liver	156.4,	55.0,	39.4

Normal figures for copper in brain and liver tissue found by Cumings are as follows (mg./100 g. dry tissue):

Cortical white matter	1.1–8.2
Cortical grey matter	2.4–9.9
Caudate	3.4–9.4
Thalamus	3.1–12.4
Putamen	6.1–12.0
Globus pallidus	10.5–18.8
Liver	3.7–17.2

Mandelbrote, Stanier, Thompson, and Thruston (1948) found that in one case of the disease which they studied there was a very high urinary excretion of copper (41.7 μg. Cu in one two-hour period) and that, as in normal persons, the administration of B.A.L. (2, 3-dimercaptopropanol) greatly increased copper excretion (109 μg. Cu in two hours). Porter confirmed the presence of increased urinary excretion

of copper in two cases of hepatolenticular degeneration and found that the administration of B.A.L. sufficient to increase the copper output by sevenfold did not significantly alter the amino-aciduria.

Quite recently Cumings (1951) has reported an investigation of four patients with hepatolenticular degeneration. In two cases showing an increased urinary output of both amino nitrogen and copper the administration of B.A.L. produced an increase in the urinary copper output, but the amino nitrogen was unaffected. These two patients improved clinically during treatment. In the two other cases, one showing a raised nitrogen and a normal copper and the other a normal amino nitrogen and somewhat raised copper output, B.A.L. had no effect on either urinary copper or amino acid excretion and neither patient improved clinically.

We present the results of an investigation of amino acid excretion and of copper metabolism in one case of hepatolenticular degeneration which came to necropsy. During life there was little evidence of liver disease, yet when the patient died hepatic cirrhosis was found. Amino acid excretion was also studied in 12 cases of other disease of the basal ganglia.

Material and Methods

Thirteen patients were investigated. These comprised one case of hepatolenticular degeneration (Case 1), four cases of Huntington's chorea, six cases of dystonia musculorum deformans, and two cases of congenital choreo-athetosis. In all of these cases amino nitrogen excretion was measured. Two of the patients suffering from dystonia (Cases 4 and 7) closely resembled clinically the fatal case of hepatolenticular degeneration. Copper excretion was investigated in Case 1 and the copper content of tissues was estimated after death.

Urinary amino nitrogen was estimated by the method of Pope and Stevens (1939) applied to urine by Albanese and Irby (1944). Twenty-four-hour collections of urine were made in bottles each containing 1 ml. of 10% alcoholic thymol and 50 ml. of approximately 1.5N hydrochloric acid. Copper in urine and tissues was estimated colorimetrically with diethyldithiocarbamate (Eden and Green, 1940). Urine for this estimation was collected in bottles that had been cleaned by washing with acid and finally with glass-distilled water. No metal was allowed to come into contact with tissues to be analysed for copper, which were cut with a sharp piece of glass, though unavoidably blood had to be taken through metal (stainless steel) needles. Serum total protein and albumin (Howe, 1921) were estimated by the micro-Kjeldahl method ; serum bilirubin with the Lovibond comparator ; serum alkaline phosphatase as described by King (1946) ; serum colloidal gold using the buffered gold sol of Maclagan (1944a) as described by Pollak (1947) ; and blood prothrombin as described by Aggeler, Howard, Lucia, Clark, and Astaff (1946), but using 0.02M calcium chloride as recommended by Quick (1945). The thymol (Maclagan, 1944b) and zinc (Kunkel, 1947) turbidity tests were also performed.

Case Notes and Results

Case 1 : Hepatolenticular Degeneration.—This boy, aged 14 years, was an only child. No significant abnormality was recorded in a full study of the family tree.* Both parents

* Including all members of the family as far back as the patient's grandparents and his grandmother's brothers.

were alive and well and showed no evidence of disease. The only previous illness of note was an attack of jaundice at the age of 7 years. The patient was of good physical and mental development until the age of 10 years. He then began to find difficulty in using his arms and legs properly : " he kept on falling about." He became hysterical at times. His movements were clumsy and tremulous and eventually he could not write. He found speaking increasingly difficult and he could not close his mouth. At times he had severe pains in the shoulders and arms. On examination he presented the typical features of the disease. He was emotional and showed the characteristic grinning expression with tight mouth and bared teeth. There was a well-marked Kayser-Fleischer ring in each eye (Fig. 1), speech was practically incoherent and writing

FIG. 1.—Kayser-Fleischer ring in hepatolenticular degeneration (Case 1).

illegible, and all voluntary movements were performed grotesquely and with much uncontrollable tremor and agitation. There were frequent powerful twisting movements of the trunk and upper limbs. At times these caused him to perspire profusely and cry out in pain. The left arm was usually held in a flexed posture behind his back and the left leg was flexed at hip, knee, and ankle. There was no significant abnormality of power or reflex activity and sensibility was normal. No enlargement of liver or spleen was perceived. His condition rapidly deteriorated while he was in hospital and there were crops of petechiae and purpuric patches over the arms and legs. At times he went into powerful tetanic muscle spasms and his body became drenched with sweat. Opisthotonos was severe at times ; speaking and swallowing were impossible. He died in such a " crisis " associated with hyperpyrexia (Figs. 2, 3, and 4). Biochemical findings are tabulated as follows :

FIG. 2.—Case 1 at the age of 13 years.

Urine.—Albumin varied from a trace to a fairly large amount; no sugar; no trace of bile pigment. Amino-aciduria was found. The patient's parents showed normal amino nitrogen excretion (Table I).

TABLE I

AMINO ACID EXCRETION IN 13 CASES OF DISEASE OF THE BASAL GANGLIA

Diagnosis	Case No.	Urinary Amino Nitrogen Excretion* (mg./24 hr.)
Hepatolenticular degeneration	1	1,005
Dystonia	2	260
,, ...	3	143
,, ...	4	146
,, ...	5	185
,, ...	6	205
,, ...	7	300
Huntington's chorea ..	8	147
,, ,, ...	9	394
,, ,, ...	10	370
,, ,, ...	11	112
Athetosis	12	245
,, ...	13	256
Normals†	14–22	232–438 (av. 332)

* In some cases the figure given is the average of more than one estimation.
† Two of these were the patient's parents: amino N. 386 and 245 respectively.

Renal Function.—Urea clearance was 107% of the average normal.

Blood.—Urea 32 mg./100 ml.; serum calcium 9.8 mg./100 ml.; platelets 260,000; clotting time (Lee and White) 3 min. 45 sec. (normal); bleeding time (Duke) 3 min. (normal); serum total protein 6.7 g./100 ml.; serum total albumin 4.45 g./100 ml.; serum alkaline phosphatase 24.7, 22.0 King-Armstrong units: serum bilirubin 0.2 mg./100 ml.; thymol turbidity 2 units

FIGS. 3 and 4.—Powerful generalized muscular spasms in terminal stages of illness (Case 1).

(repeatedly); Kunkel turbidity 8, 7 units; serum colloidal gold 000000; prothrombin 80% of average normal; blood copper 80 μg./100 ml.

The liver function tests, which were all done within a month of the patient's death, gave normal results except that the phosphatase value was rather high: Sunderman (1942) gives the range of normal values for children as 15–20 King-Armstrong units.

At the post-mortem examination the surface of the liver was found to be nodular (Fig. 5). A large section was prepared and this showed numerous nodules, most of which were about 1 cm. in diameter, with intervening fibrosis (Fig. 6). This section clearly shows that a punch biopsy might not reveal cirrhosis, as it would be quite easy to take the specimen from the centre of one of these nodules. Microscopy revealed cirrhosis (Fig. 7). The spleen was enlarged (300 g.). There was much proliferation of the endothelium of the sinuses and the follicles were atrophic. In the central nervous system, only the putamen was abnormal, showing neuronal degeneration. Acute bronchitis was present in the lungs. The other organs, including the kidney, appeared normal.

After death it was found that the copper content of brain and liver tissue was raised, as shown in Table II. Liver tissue obtained from a woman who died as a result of a head

TABLE II

COPPER CONTENT OF TISSUES IN HEPATOLENTICULAR DEGENERATION

Tissue	Copper (mg./100 g. dry tissue)	Water (%)
Liver (left lobe)	37	75·3
Liver (right lobe)	33	73·5
Spleen	about 1	79·6
Kidney	28	78·7
Cortical white matter	18	70·3
Cortical grey matter	37	83·2
Caudate	27	86·3
Thalamus	60	80·5
Putamen	37	87·2
Globus pallidus	17	79·2

injury was found to contain only 2 mg. of copper per 100 g. of dried tissue. Normal values for brain and liver tissue found by Cumings are given on p. 17. In confirmation of the findings of Mandelbrote and his colleagues and of Porter we found that in our case of hepatolenticular degeneration B.A.L. caused an increased excretion of copper in the urine (Table III).

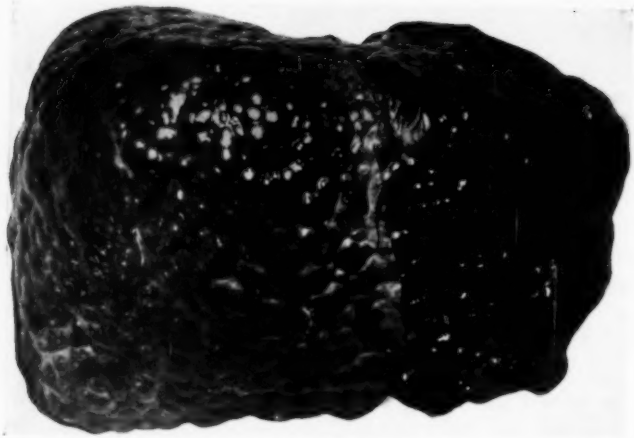
TABLE III

EFFECT OF B.A.L. ON URINARY COPPER EXCRETION IN HEPATOLENTICULAR DEGENERATION

Period	Volume (ml.)	Copper Content (μg.)
1 (2½ hr.)	84	76
2 (3 hr.)	140	98
100 mg. B.A.L. injected at end of second period		
3 (2½ hr.)	123	192
4 (1¼ hr.)	63	46

Case 4: Dystonia.—A boy, aged 6 years, was well until 3 years of age, then had a series of convulsions. For two years "his right hand began to go behind his chest"

FIG. 5



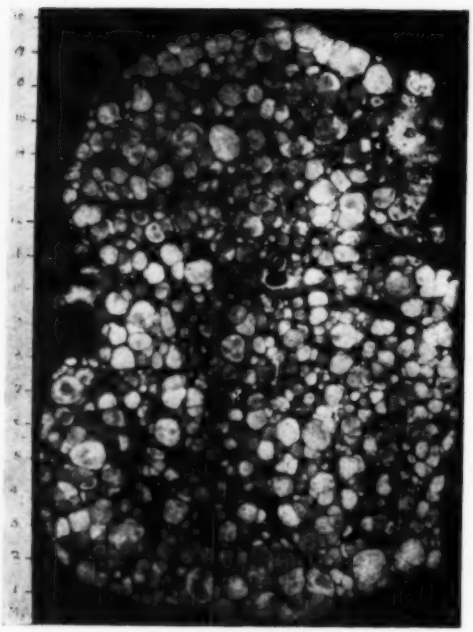


FIG. 6

FIG. 5.—Gross appearance of liver (Case 1).

FIG. 6.—Section of liver of Case 1 prepared by method of Gough and Wentworth (1949).

FIG. 7.—Histological appearance of liver (Case 1).

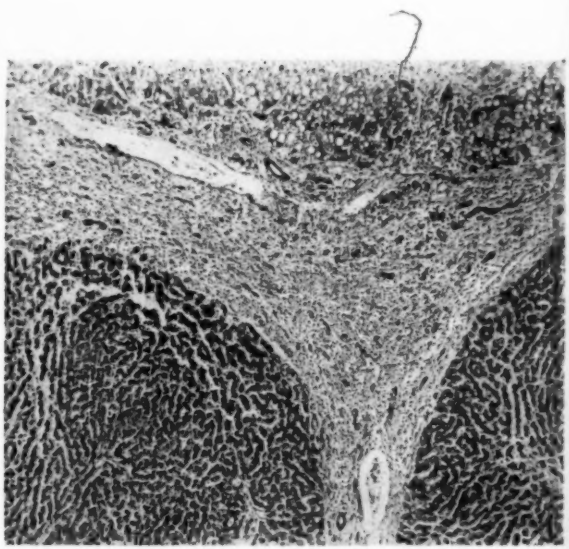


FIG. 7

(mother's statement). Then later, " his right foot went funny." He became excitable and difficult. Intelligence appeared normal, but he did not go to school. Speech was normal ; he walked in a jerky, " dancing " fashion, with abrupt flexion movements of the right leg. The right hand was flexed behind his back, and he showed occasional twisting movements of the trunk, and generalized tremors. Grimacing and giggling were frequently noted. There was no evidence of liver disease, the eyes were normal, and no history of jaundice was elicited. His behaviour and stance were similar to those of Case 1. An air encephalogram was normal. Muscular hypertonicity on the right and left sides, more marked on the right, was present. Amino acid excretion was normal. The blood copper level was normal. Renal function tests were normal.

Case 7 : Dystonia.—This woman, aged 32 years, had a normal family history. She had jaundice at the age of 10 years. Since 11 years she has had progressive dystonia culminating in generalized muscular rigidity, with incoherent speech, dysphagia, generalized tremors, and powerful torsion movements of the neck, trunk, and limbs. She was very emotional, and would laugh and cry with great ease and readiness. The facial appearance and history of jaundice raised the possibility of hepatolenticular degeneration. Her face was set in a " smiling " manner as in Case 1. Renal and liver function tests were normal. The blood copper level was normal. Liver biopsy was performed, but there was insufficient material obtained for adequate examination. Amino acid excretion was normal.

TABLE IV

BLOOD AND URINE COPPER EXCRETION IN SIX CASES

Case No.	Blood Copper (μg./100 ml.)	Urine Copper (μg./24 hours)
1	80	760*
2	115	40
3	105	20
4	135	—
5	90	30
13	100	60

* Calculated from 5½-hr. period (see Table III).

Comment

It was confirmed that in this series only in the patient with hepatolenticular degeneration was there a disturbance of amino acid excretion. In this case liver function tests revealed no abnormality (with the exception of the raised phosphatase) yet when the patient died severe hepatic cirrhosis was present. This suggests that in attempting to explain the origin of the amino-aciduria in this disease the liver must not be assumed to be normal because such tests give normal results. In contrast to eight of the nine cases reported by Sweet, Gray, and Allen (1941) our case of hepatolenticular degeneration showed a normal serum colloidal gold reaction. These results as a whole might have been interpreted as indicating that in our case the amino-aciduria pointed to some constitutional metabolic error, and was not a result of liver disease.

The absence of amino-aciduria may be helpful in the diagnosis of doubtful cases, as in our Cases 4 and 7. Both patients were facile and emotional ; they giggled and smiled abnormally. In Case 4 there was the same complaint that " the right arm goes behind his back all the time." In Case 7 there was a history of jaundice and

the facial appearance was not unlike that of hepatolenticular disease. The copper concentration of the blood was normal in each case.

The significance of the disturbance of copper metabolism in hepatolenticular degeneration remains a mystery.

Summary

Urinary amino nitrogen excretion was measured in 13 people with disease of the basal ganglia and in nine normal people. It was raised only in a boy with hepatolenticular degeneration, neither of whose parents had amino-aciduria.

In this case, liver function tests in the month before death showed little evidence of liver disease. At necropsy hepatic cirrhosis was found.

This patient excreted large amounts of copper in the urine. Administration of B.A.L. increased the copper output.

Brain and liver tissue obtained at necropsy contained excessive amounts of copper.

We wish to thank the following: Messrs. G. R. Armstrong and H. A. Griffiths for the photographs, Miss Nansi Gwynne for the painting of the eye, Dr. A. G. Heppleston for the post-mortem examination, Mr. J. E. Wentworth for preparing the large section of the liver, and Professor J. Gough for his help and encouragement.

References

Aggeler, P. M., Howard, J., Lucia, S. P., Clark, W., and Astaff, A. (1946). *Blood*, **1**, 220.
Albanese, A. A., and Irby, V. (1944). *J. biol. Chem.*, **153**, 583.
Ames, S. R., and Risley, H. A. (1948). *Proc. Soc. exp. Biol., N.Y.*, **68**, 131.
Cooper, A. M., Eckhardt, R. D., Faloon, W. W., and Davidson, C. S. (1950). *J. clin. Invest.*, **29**, 265.
Cumings, J. N. (1948). *Brain*, **71**, 410.
—— (1951). Ibid., **74**, 10.
Eckhardt, R. D., Cooper, A. M., Faloon, W. W., and Davidson, C. S. (1948). *Trans. N.Y. Acad. Sci.*, **10** (Series 2), 284.
Eden, A., and Green, H. H. (1940). *Biochem. J.*, **34**, 1202.
Glazebrook, A. J. (1945). *Edinb. med. J.*, **52**, 83.
Gough, J., and Wentworth, J. E. (1949). *J. roy. micr. Soc.*, **69**, 231.
Haurowitz, F. (1930). *Hoppe-Seyl. Z.*, **190**, 72.
Howe, P. E. (1921). *J. biol. Chem.*, **49**, 93.
King, E. J. (1946). *Micro-analysis in Medical Biochemistry*. London.
Kunkel, H. G. (1947). *Proc. Soc. exp. Biol., N.Y.*, **66**, 217.
Maclagan, N. F. (1944a). *Brit. J. exp. Path.*, **25**, 15.
—— (1944b). Ibid., **25**, 234.
Mandelbrote, B. M., Stanier, M. W., Thompson, R. H. S., and Thruston, M. N. (1948). *Brain*, **71**, 212.
Pollak, H. (1947). In *Recent Advances in Clinical Pathology*, ed. S. C. Dyke, p. 147. London.
Pope, C. G., and Stevens, M. F. (1939). *Biochem. J.*, **33**, 1070.
Porter, H. (1949). *J. Lab. clin. Med.*, **34**, 1623.
Quick, A. J. (1945). *Amer. J. clin. Path.*, **15**, 560.
Sunderman, F. W. (1942). Ibid., **12**, 404.
Sweet, W. H., Gray, S. J., and Allen, J. G. (1941). *J. Amer. med. Ass.*, **117**, 1613.
Uzman, L., and Denny-Brown, D. (1948). *Amer. J. med. Sci.*, **215**, 599.

J. clin. Path. (1952), **5**, 25.

THE NITROPRUSSIDE METHOD FOR THE DETERMINATION OF BLOOD GLUTATHIONE

BY

R. H. S. THOMPSON AND D. WATSON

From the Department of Chemical Pathology, Guy's Hospital Medical School, London

(RECEIVED FOR PUBLICATION JUNE 18, 1951)

Recent studies on alloxan diabetes (Lazarow, 1949), together with observations on the effect of adrenocorticotropic hormone on available sulphydryl compounds (Conn, Louis, and Johnston, 1949), have focused the attention of a number of laboratories on the level of blood glutathione in diabetes and other conditions.

In the course of work in this laboratory on the effects produced in the body by various heavy metals the need arose for the estimation of blood glutathione levels by some method other than an iodometric one. Brückmann and Wertheimer's (1947) modification of the nitroprusside method described by Fujita and Numata (1939) was therefore adopted, but was not found to give consistent results in our hands. A number of methods for the determination of glutathione (GSH) in blood and tissues by means of the nitroprusside reaction have been described in the literature, but most of these have suffered from a rapid fading of the final colour and, in some instances, from the instability of one or more of the reagents. For these reasons the various steps in the procedure of Brückmann and Wertheimer (1947) have been examined in detail. A modified method has been worked out which is simple and has the double advantage of giving greater sensitivity and also greater stability of the final colour.

Experimental

Reagents.—The following were used.

(1) 10% (w/v) trichloroacetic acid (AR).

(2) Ammonium sulphate, solid (AR).

(3) Ammonium sulphate, saturated aqueous solution.

(4) M-sodium nitroprusside (AR), 0.05 in saturated $(NH_4)_2SO_4$.

(5) 8 N Ammonium hydroxide (AR).

(6) Standard glutathione (B.D.H.) solution, 1 mg./ml. This solution must be freshly prepared for each estimation.

All solutions must be prepared with glass-distilled water, and all glassware must be scrupulously clean.

Method.—A quantity, 0.5 ml., of freshly drawn blood (oxalated or heparinized) is added to 1.5 ml. of glass-distilled water in a 15 ml. centrifuge tube, and allowed to stand for five minutes to lake. Then 2 ml. of 10% trichloroacetic acid are added, and the mixture well stirred with a glass rod and allowed to stand for five minutes, after which it is centrifuged for five minutes at 3,000 r.p.m. Then 2 ml. of the clear, protein-free supernatant are pipetted into a stoppered 10 ml. graduated cylinder. Solid $(NH_4)_2SO_4$ is added to saturate the solution (by tipping in 1.4 g. contained in a marked tube); after shaking the volume is made up to 3 ml. with saturated $(NH_4)_2SO_4$.

For the production of the colour 0.5 ml. of 0.05 M-nitroprusside reagent is added, followed by 0.7 ml. of 8N NH_4OH.

After transferring into cells of 1 cm. optical depth the colour intensity is read in a photoelectric colorimeter 30 seconds after the addition of the NH_4OH, using an Ilford spectrum green (604) filter. A reagent blank determination is carried out using 1 ml. water and 1 ml. 10% trichloroacetic acid, with all subsequent additions as for the blood filtrate. All tubes are compared against water.

Standards.—The standard glutathione solution is diluted tenfold with H_2O to give a solution containing 100 μg. GSH/ml. To prepare a standard curve 0.2, 0.4 . . . 1.0 ml. samples (i.e. 20, 40, . . . 100 μg. GSH) are each diluted with H_2O to 1.0 ml. Then 1.0 ml. of 10% trichloroacetic acid is added to each, and the estimation carried out as for the blood filtrates. One standard tube (containing 60 or 80 μg. GSH) is included in each series of blood estimations.

Results

We have avoided the use of saponin and cf saturation with NaCl before precipitation of proteins as in the method of Brückmann and Wertheimer (1947), because of the difficulty which we experienced in obtaining clear, protein-free supernatants without recourse to filtering. By initially diluting the blood with 1.5 ml. of water an adequate degree of laking is obtained ; centrifugation after precipitating the proteins with trichloroacetic acid then results in a well-packed precipitate with a clear supernatant. The use of ammonium sulphate rather than NaCl to saturate this supernatant results also in greater colour intensities for a given concentration of GSH.

Fujita and Numata (1939) and Brückmann and Wertheimer (1947) used 1% sodium nitroprusside in 0.4% $(NH_4)_2SO_4$. This reagent is extremely unstable, whereas 0.05 M (1.5%) sodium nitroprusside in saturated $(NH_4)_2SO_4$ is stable for at least a week. It was also found to give greater intensities than the 1% solution. We have also used 8N NH_4OH instead of the 25% Na_2CO_3 recommended by Brückmann and Wertheimer (1947) on account of the greater optical density produced (E for 60 μg. GSH = 0.130 with 25% Na_2CO_3, 0.170 with 35% K_2CO_3, and 0.295 with 8N NH_4OH).

The results which we have obtained with the method described above using pure solutions of GSH are shown in Table I, mean values and standard deviations being given for the extinctions obtained in 10 different experiments.

It will be seen that the colour intensities follow Beer's law for amounts up to 100 μg. Higher quantities have not been studied, since blood levels do not normally exceed this range.

TABLE I

OPTICAL DENSITIES OF COLOURS DEVELOPED FROM AQUEOUS SOLUTIONS OF GSH *

	GSH (μg.)				
	20	40	60	80	100
Mean optical densities ..	0·093	0·187	0·281	0·377	0·467
S.E.	±0·005	±0·005	±0·008	±0·009	±0·006

* Readings at 1 cm. cell depth using an Ilford green filter 604. Ten observations were made at each concentration.

The variability from day to day in the results obtained with these standard solutions is not large, and is almost certainly due chiefly to changes in room temperature. We have confirmed the findings of Bierich and Rosenbohm (1933) and Grunert and Phillips (1951) that the colour intensity decreases with increasing temperature ; it is therefore advisable to carry out the estimations at a standard temperature, although, if, as is recommended, a standard solution of GSH is included in each series of blood estimations, the final error due to temperature changes is not great.

Stability of Colour.—Under the final conditions chosen the rate of fading of the colour up to 50–60 seconds is sufficiently low to permit reasonably accurate reading at 30 seconds after the addition of the NH_4OH. The optical densities (uncorrected for the blank) obtained at varying intervals up to 90 seconds are shown in Table II.

TABLE II

OPTICAL DENSITY OF SOLUTIONS AT INTERVALS AFTER ADDITION OF NH_4OH *

GSH (μg.)	Uncorrected Optical Densities at					
	20	30	40	50	60	90 sec.
0	0·071	0·071	0·073	0·077	0·080	0·083
25	0·186	0·184	0·184	0·185	0·187	0·177
50	0·316	0·311	0·308	0·305	0·302	0·290
75	0·442	0·439	0·432	0·424	0·418	0·401
75	0·445	0·440	0·435	0·428	0·420	0·390
100	0·532	0·525	0·516	0·508	0·500	0·461
100	0·530	0·520	0·513	0·510	0·505	0·475

* Readings at 1 cm. cell depth using an Ilford green filter 604. Ten observations were made at each concentration.

Recovery of Added GSH.—To assess the accuracy of the method when applied to blood, recovery experiments have been carried out with known amounts of GSH added to samples of blood immediately after drawing and before precipitation of proteins (Table III).

TABLE III

RECOVERY OF GSH ADDED TO BLOOD SAMPLES IMMEDIATELY AFTER DRAWING

GSH (μg.) in Original Blood Sample	GSH (μg.) Added	GSH (μg.) Found	GSH (μg.) Recovered	Recovery (%)
70·0	10	79·6	9·6	96
75·5	15	89·4	13·9	93
80·2	15	95·0	14·8	99
77·6	20	96·6	19·0	95
60·5	20	80·0	19·5	98

Estimation of GSH in Blood.— The GSH contents of samples of blood from 21 healthy adults (15 male and six female) have been determined by this method. No significant difference was found between the values for males and females. In each case the packed cell volume was determined and found to be within the normal range. The mean value for the GSH level was 30.7 mg./100 ml. (S.E. $= \pm 2.05$).

In a study of the GSH content of various tissues Leaf and Neuberger (1947) found that the values obtained by an iodometric method were, in the case of liver and kidney, often considerably higher than those obtained by the nitroprusside method of Fujita and Numata (1939); a similar discrepancy between these two methods had been described earlier by Bierich and Rosenbohm (1933). We have therefore compared, on samples from 13 different healthy subjects, the results obtained by our method with those found by the method of Woodward and Fry (1932). In each case the nitroprusside method gave a lower value than that obtained iodometrically, the difference varying from 0.7 to 16.8 mg./100 ml.

TABLE IV

COMPARISON OF BLOOD GSH LEVELS IN 13 DIFFERENT SUBJECTS BY NITROPRUSSIDE AND IODOMETRIC METHODS

Nitroprusside (mg. GSH/100 ml.)	Iodometric (mg. GSH/100 ml.)	Difference
32·1	34·6	−2·5
30·0	30·7	−0·7
35·4	37·4	−2·0
29·3	41·8	−12·5
31·6	35·1	−3·5
30·1	36·8	−6·7
27·9	31·6	−3·7
29·4	32·0	−2·6
31·6	35·2	−3·6
31·7	48·5	−16·8
30·8	34·6	−3·8
33·4	34·3	−0·9
32·5	40·2	−7·7

Discussion

One of the chief disadvantages in the use of the nitroprusside reaction for the quantitative determination of thiols has been the instability of the colour. Although the observed rate of fading in the method described here is small a few experiments were done in the hope of minimizing this. Abderhalden and Wertheimer (1923) had reported that the addition of cyanide decreased the rate of fading, and in view of the reactivity of thiols with traces of metals we therefore investigated the effect of the addition of KCN and of various metal-binding agents before developing the colour. Neither KCN, 8-hydroxyquinoline, sodium diethyldithiocarbamate, nor α-benzoin oxime ("cuppron") had any observed effect in the concentrations studied. This finding agrees in general with the results of Herrmann and Moses (1945), who also studied the effect of various metal-binding agents, although these workers did report some stabilization with hydroxyquinoline.

The trichloroacetic acid extracts can safely be left for at least one hour at room temperature before carrying out the nitroprusside reaction without subsequent loss of colour intensity. In agreement with Leaf and Neuberger (1947) we have found that if the NH_4OH is not added immediately after the addition of the nitroprusside reagent the colour intensity produced is low.

Unless the reaction is carried out at a carefully controlled temperature, it is inadvisable to attempt to work from any precalibrated standard curve ; a standard GSH solution should, instead, be included in each series of estimations.

Since some workers are now interested in blood GSH levels in diabetes, we felt it desirable to know whether any interference was to be expected, under the conditions of our method, from the slowly developing nitroprusside reaction given by aceto-acetic acid. The level of total ketone bodies in the blood in health is not usually much in excess of 1 mg./100 ml. expressed as β-hydroxybutyric acid (Weichselbaum and Somogyi, 1941 ; Greenberg and Lester, 1944 ; Billing, 1951). Amounts of aceto-acetic acid roughly equivalent to blood levels of 4 mg./100 ml. gave no measurable colour at 30 seconds, although by 90 seconds a significant degree of colour had already developed ; amounts of aceto-acetic acid much above this level would of course interfere even at 30 seconds.

Summary

The nitroprusside method for the estimation of blood glutathione has been studied, and modifications made in order to increase the sensitivity and decrease the rate of fading of the final colour.

Our thanks are due to the volunteers who provided blood samples in the course of this work, and to Mr. D. H. Burchett for technical assistance.

REFERENCES

Abderhalden, E., and Wertheimer, E. (1923). *Pflüg. Arch. ges. Physiol.*, **198**, 122.
Bierich, R., and Rosenbohm, A. (1933). *Hoppe-Seyl. Z.*, **215**, 151.
Billing, B. H. (1951). Unpublished results.
Brückmann, G., and Wertheimer, E. (1947). *J. biol. Chem.*, **168**, 241.
Conn, J. W., Louis, L. H., and Johnston, M. W. (1949). *J. Lab. clin. Med.*, **34**, 255.
Fujita, A., and Numata, I. (1939). *Biochem. Z.*, **300**, 246.
Greenberg, L. A., and Lester, D. (1944). *J. biol. Chem.*, **154**, 177.
Grunert, R. R., and Phillips, P. H. (1951). *Arch. Biochem.*, **30**, 217.
Herrmann, H., and Moses, S. G. (1945). *J. biol. Chem.*, **158**, 33.
Lazarow, A. (1949). *Physiol. Rev.*, **29**, 48.
Leaf, G., and Neuberger, A. (1947). *Biochem. J.*, **41**, 280.
Weichselbaum, T. E., and Somogyi, M. (1941). *J. biol. Chem.*, **140**, 5.
Woodward, G. E., and Fry, E. G. (1932). Ibid., **97**, 465.

J. clin. Path. (1952), **5**, 30.

THE DETERMINATION OF BLOOD GLUTATHIONE BY AMPEROMETRIC TITRATION

BY

D. S. BIDMEAD AND D. WATSON

From the South-west and Oxford Regions Rheumatism Research Unit, Royal National Hospital for Rheumatic Diseases, Bath

(RECEIVED FOR PUBLICATION SEPTEMBER 5, 1951)

In 1946 Kolthoff and Harris devised a method of titrating mercaptans in aqueous alcoholic solution with silver nitrate. They used a rotating platinum electrode with suitable current measuring equipment to locate the titration end-point. Benesch and Benesch (1948) applied this technique to the determination of sulphydryl groups in serum protein, and Weissman, Schoenbach, and Armistead (1950) used the same method in a study of the sulphydryl content of albumin and globulin fractions in normal sera.

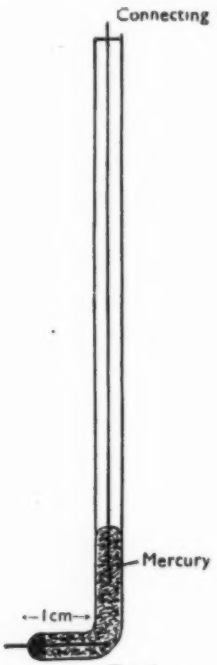


FIG. 1

Since variations in the sulphydryl concentration of the blood are of interest to workers in the field of rheumatic diseases and to those concerned with diabetes, it was considered that a relatively simple method of estimating blood "glutathione" might usefully be evolved from an amperometric titration procedure. This proved to be correct. A description of the apparatus, an account of the technique, and of the results obtained, are now presented.

Experimental

Apparatus.—The apparatus follows the design of Kolthoff and Harris (1946) with certain modifications, and consists essentially of a rotating electrode, a reference half-cell, a salt-bridge, a micro-ammeter or galvanometer, and a microburette.

Rotating Electrode (*A*).—The electrode consists of about 15 cm. of 5 mm. bore soft-glass tubing bent at right angles at one end to give approximately 1 cm. length in the bent section. Into the angle piece is fused a platinum wire 0.5 mm. thick, so that 6 mm. length of wire protrudes from the end of the tube, and the free end of the wire lies inside the glass tube. Contact is made by pouring a small quantity of mercury into the glass tube (Fig. 1). The glass electrode is fitted into a hollow shaft surmounted by a pulley running in a vertical bearing and driven by a small electric motor. The pulley wheel is attached to the shaft

and has a central hole so that a wire can be passed through the rotating shaft into the mercury contact at the bottom of the electrode.

Reference Half-Cell (*B*).—This is a mercury mercuric iodide cell having a potential of –0.23 volt against the saturated calomel electrode. It is set up in the type of glass container used for calomel electrodes fitted with a side-arm and stopcock. A layer of mercury serves as the electrode, and sufficient should be added to give the maximum surface area in order to prevent polarization. The remainder of the cell, including its side-arm, is filled with electrolyte, which consists of 4.2 g. of potassium iodide and 1.3 g. of mercuric iodide dissolved in 100 ml. of saturated potassium chloride solution.

Salt-Bridge (*C*).—The side-arm of the half-cell dips into a beaker containing saturated potassium chloride. A piece of 6 mm. glass tubing approximately 60 cm. long is filled with a gel of 3% agar in saturated potassium chloride and is bent into a rough inverted U-shape, so that one end dips into the beaker of potassium chloride solution and the other into the beaker containing the solution under titration. The actual shape of the tube depends entirely upon the particular assembly in use. The agar bridge lasts for some months before renewal is necessary. Shrinkage of the agar does not interfere with the conductivity, provided that no cracks or splits occur. During use the electrolyte in the reference half-cell slowly diffuses into the beaker of saturated potassium chloride and vice versa. When this has occurred to some extent, the galvanometer response may be sluggish and weak. Renewal of the electrolyte usually remedies this. The top surface of the beaker containing saturated potassium chloride, the whole of the side-arm of the reference half-cell, and about 10 cm. of the end of the agar-bridge dipping into the beaker should be thoroughly greased with vaseline to prevent the potassium chloride "creeping," as this may cause other electric potentials to be set up which interfere with the normal galvanometer readings.

Current Measuring Device (*D*).—A reliable micro-ammeter registering 0–10 micro-amp. in divisions of 0.2 micro-amp. has been found to give excellent results. A Cambridge spot galvanometer, 450 ohms, is also satisfactory when used with a variable shunt resistance of 5,000 ohms, although, due to the greater sensitivity of the galvanometer, the readings are not so precise as those of the micro-ammeter. In general, the lowest sensitivity consistent with accurate readings gives the best results.

The Micro-burette (*E*).—A 1 ml. self-filling burette with three way stopcock graduated in 0.01 ml. is used, with a drawn-out tip so that one drop measures not more than 0.01 ml. This gives a slow running time to the burette and enables 0.1 ml. aliquots to be run out accurately.

Assembly of Apparatus.—By means of a wire making contact with the mercury, the rotating electrode is connected to the positive terminal of the micro-ammeter; the reference electrode is wired to the negative terminal. The micro-burette is secured in a clamp so that the delivery tip is as close as possible to the rotating electrode without interfering with its movement. Another clamp holds the agar salt-bridge, one end of which is placed close to and approximately level with the platinum electrode, while the other end dips into the saturated potassium chloride. As described above, the side-arm of the reference half-cell also dips into the beaker of potassium chloride. The circuit is completed by the solution to be titrated, which is contained in a 150 ml. beaker held in a retort clamp so arranged that by simply raising or lowering the beaker the contents make or break the circuit between the rotating electrode and the agar bridge. Thus all other parts of the circuit are fixtures, but the 150 ml. beaker is changed for each titration (Fig. 2).

Method.—A protein-free trichloracetic acid extract of blood is titrated with silver nitrate, and the progress of the reaction of sulphydryl compounds with Ag ions followed

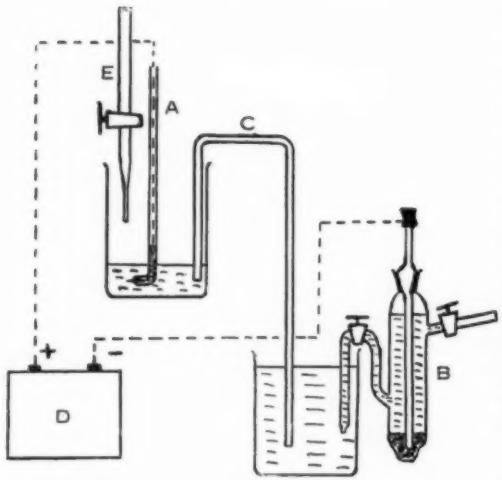


FIG. 2

amperometrically. With a suitable potential difference, silver is electro-reduced and a diffusion current flows between the two electrodes. In a supporting electrolyte of ammonia and ammonium nitrate the initial addition of silver causes precipitation of thiols as silver mercaptide (RSAg), after which further additions produce a progressively rising current proportional to the ionic concentration of silver. Chloride does not interfere with the titration. By incorporating alcohol in the reaction mixture, the decreased solubility of silver mercaptide allows a more clear-cut end-point to be obtained.

Technique.—Whole blood, 1 ml., freshly drawn into an all-glass syringe is laked with 3 ml. of glass-distilled water in an acid-washed centrifuge tube. After five minutes 4 ml. of 10% trichloracetic acid are added and well mixed. The tube is then centrifuged at 3,000 revs. for five minutes.

In a clean 150 ml. beaker, 21 ml. of 0.06 M ammonium nitrate solution are mixed with 4 ml. of pure ethyl alcohol and 4 ml. of the supernatant fluid from the centrifuge tube. The beaker is now clamped in position so that the electrode and the end of the salt-bridge are covered by the contents. The electrode is set rotating and the side-arm stop-cock of the reference half-cell is opened. The micro-ammeter should register a relatively high current (6–8 micro-amp.). Whilst the electrode is rotating, two drops (0.1 ml.) of ammonia solution (sp. gr. 0.880) are carefully added. On addition of the second drop the micro-ammeter needle should quickly return to a minimum value. This value varies from time to time, depending upon the number of previous estimations, if any, and the state of the reference half-cell, but it is usually from 0.4–1.0 micro-amp. Immediately after adding the

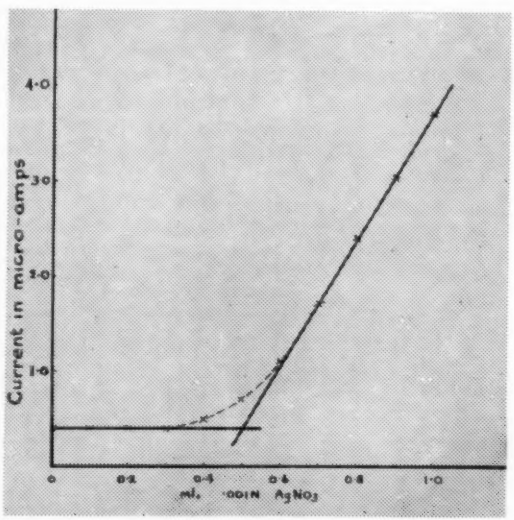


FIG. 3

ammonia, the titration is begun : 0.001 N silver nitrate is run into the beaker 0.1 ml. at a time, the reading of the micro-ammeter being noted after each addition. The readings remain stationary for the first few additions of silver nitrate and near the end-point begin to increase. About six more readings are taken after the initial movement.

Duplicate titrations should be made on each test sample. Before beginning a series of determinations a blank titration of 25 ml. of ammonium nitrate solution, 4 ml. of alcohol, and 0.1 ml. of ammonia should be made to enable the apparatus to be checked for satisfactory performance. The micro-ammeter response should be almost immediate, and the blank titration will not be more than 0.05 ml. of 0.001 N silver nitrate.

The end-point is found graphically (Fig. 3) by plotting the diffusion current against the volume of silver nitrate. One line can be drawn through the stationary readings of the micro-ammeter and a second straight line through the increasing readings. At the point where these lines intersect the corresponding volume of silver nitrate is read, and this volume multiplied by 200 gives the number of micromoles sulphydryl per 100 ml. of blood. Conversion to glutathione (mg. per 100 ml.) is readily obtained by multiplying μM sulphydryl per 100 ml. by 0.307.

General Observations.—After about 30 titrations have been carried out the platinum electrode becomes slightly coated with silver, with consequent loss of sensitivity. However, the efficiency of the electrode is easily restored by cleaning it in nitric acid when necessary.

The reaction mixture should have a *p*H of 8.5–9.0 ; excess ammonia must *not* be added. If the titration is delayed after adding 0.1 ml. ammonia low results will be obtained. The silver nitrate solution (0.001 N) is best prepared freshly each day from a 0.1 N solution contained in a dark bottle. Industrial methylated spirit may be used in place of ethyl alcohol, provided that the spirit has not at any time been stored in metal containers. All reagents must be free from traces of iron and copper.

Results

To test the accuracy of the method, the following procedures were carried out. Eight aqueous solutions containing amounts of glutathione (GSH) unknown to the operator were titrated. The mean percentage recovery was 96 (range, 91–98%). Volumes of glutathione solutions chosen at random and representing 10, 20, or 30 mg. GSH per 100 ml. of blood were added to laked blood samples of known sulphydryl content ; the amount of GSH then estimable was found by titration. In 40 such experiments the mean recovery of added GSH was 98.4% (S.D. ± 4.3%).

Table I presents values for the glutathione content of 45 different samples of blood as determined by silver titration, by the iodometric assay of Woodward and Fry (1932), and by a recently improved nitroprusside reaction (Thompson and Watson, 1952).

The sulphydryl groups of ergothioneine hydrochloride and cysteine hydrochloride are, as expected, estimable by silver titration. Ergothioneine hydrochloride is also recoverable when added to protein-free blood filtrates. When, however, this substance is added to blood before its laking with water, ergothioneine is not titratable under our experimental conditions, and is apparently removed with the protein precipitate.

Discussion

The values given in Table I are comparative only and do not necessarily represent the true *in vivo* blood GSH levels. Nevertheless it will be seen that results obtained by the colorimetric nitroprusside method and by the method now advocated are in good agreement, while those obtained by the iodometric procedure are high.

TABLE I

COMPARISON OF GLUTATHIONE VALUES OF 45 WHOLE BLOODS BY THREE METHODS

Present Method	Thompson and Watson (1952)	Woodward and Fry (1932)
36·0*	39·1	41·4
35·6	34·2	40·2
42·3	38·8	39·7
32·9	31·9	34·2
27·6	25·0	28·5
41·8	42·1	37·1
30·7	32·7	32·8
30·7	34·0	34·3
40·6	44·0	46·8
33·8	37·0	44·4
31·7	39·0	35·7
33·1	35·0	31·3
32·0	36·5	35·7
31·9	30·6	33·4
35·6	29·6	38·4
44·7	42·0	48·3
34·1	30·0	32·7
36·8	32·0	35·6
33·1	27·0	32·7
35·6	29·3	32·7
31·9	26·5	29·9
35·0	32·4	35·6
35·0	36·7	38·4
29·2	28·8	31·3
28·9	32·2	35·6
27·0	27·2	32·8
27·0	27·0	28·5
32·5	34·3	41·4
28·8	31·1	32·8
26·4	26·5	29·9
33·2	33·8	42·7
32·5	32·5	44·0
33·1	32·9	38·4
29·4	29·8	29·9
39·9	38·8	44·2
24·5	26·2	27·0
25·8	29·2	28·5
31·3	33·6	35·6
25·8	30·0	31·3
33·0	36·7	44·0
25·2	28·3	39·9
22·1	22·7	27·0
30·7	30·2	38·4
27·6	27·6	35·6
22·1	22·4	28·5
Mean of results 32·0	32·2	35·5
Standard deviation ±5·1	±5·1	±5·1

* Results expressed in mg. GSH per 100 ml.

Woodward and Fry (1932) tested their iodometric analysis for blood glutathione for possible interference by certain non-thiol reducing substances present in blood filtrates. Although they apparently did not consider the presence of ascorbic acid,

one substance, ergothioneine, was shown to be the cause of an error to the extent of about 3 mg. per 100 ml. blood. By the present technique ergothioneine is not estimated, a fact which might account for the discrepancy between the results obtained by these two methods. While it is possible that other non-protein sulphydryl compounds, e.g. peptides of cysteine, may contribute both to a nitroprusside colour and to the formation of a silver salt, it is clear from our results that iodometric titration is a less specific determinative procedure for blood glutathione.

Since it is known that oxidized thiols do not give the nitroprusside colour reaction, the agreement shown by the nitroprusside and present procedures suggests that disulphides do not interfere with our estimation of glutathione. In view of work by Cecil (1950) on the potentiometric analysis of aqueous thiols and disulphides, a few analyses were made on a specimen of blood to some samples of which oxidized glutathione (G.S.S.G.) had been added. There was virtually no difference in the calculated glutathione content of the samples. Hydrolytic decomposition of oxidized glutathione does not therefore occur during the titration of glutathione.

Summary

A simple and rapid technique is described for the determination of blood glutathione.

Results are given for recoveries of glutathione added to blood of known sulphydryl content, and for the estimation of blood non-protein sulphydryl compounds carried out in parallel by iodometric and nitroprusside methods, and by a silver nitrate titration.

Titration with Ag ions to an amperometrically found end-point is shown to provide a more accurate, specific, and reliable method for the determination of reduced glutathione in blood.

The authors wish to thank Dr. G. D. Kersley, director of the Rheumatism Research Unit, for a helpful discussion on this work, and are also grateful to Dr. H. J. Gibson, Royal National Hospital for Rheumatic Diseases, Bath, for the provision of many of the blood samples.

We are indebted to Messrs. Burroughs Wellcome and Co. for a gift of ergothioneine.

REFERENCES

Benesch, R., and Benesch, R. E. (1948). *Arch. Biochem.*, **19**, 35.
Cecil, R. (1950). *Biochem. J.*, **47**, 572.
Kolthoff, I. M., and Harris, W. E. (1946). *Industr. Engng. Chem. (Anal. ed.)*, **18**, 161.
Thompson, R. H. S., and Watson, D. (1952). *Journal of Clinical Pathology*, **5**, 25.
Weissman, N., Schoenbach, E. B., and Armistead, E. B. (1950). *J. biol. Chem.*, **187**, 153.
Woodward, G. E., and Fry, E. G. (1932). Ibid., **97**, 465.

J. clin. Path. (1952), **5**, 36.

NECROTIZING PULMONARY ARTERIOPATHY ASSOCIATED WITH PULMONARY HYPERTENSION*

BY

W. St. C. SYMMERS

From the Department of Pathology, University of Birmingham

(RECEIVED FOR PUBLICATION JULY 30, 1951)

The occurrence of pulmonary arteriosclerosis and arteriolosclerosis has long been recognized. Such structural changes in the pulmonary vasculature have usually been considered to represent an adaptation to alterations in the haemodynamics of the lesser circulation. In particular, their presence has been related to pulmonary hypertension, and they have been compared to the changes which occur in the systemic vasculature in association with hypertension of the major circulation. It is interesting that there does not appear to be a pulmonary arteriolar counterpart of the hyaline sclerosis of the smaller systemic arterioles which is so characteristic of "essential" systemic hypertension; the explanation of the non-occurrence of this type of hypertensive arteriolar change in the pulmonary vasculature is probably to be found in the structural differences which exist between pulmonary and systemic arterioles.

There are remarkably few published observations concerning the association of pulmonary hypertension with necrotizing pulmonary arteriolar lesions which might be considered to be analogous to the necrosis of systemic arterioles seen in cases of malignant systemic hypertension. Parker and Weiss (1936) studied the lungs in a series of cases of mitral stenosis. In five of these cases in which there was clinical and morbid anatomical evidence of severe embarrassment of the pulmonary circulation they observed necrotizing pulmonary arteriolitis in addition to hyperplastic arteriolosclerosis. They stressed the similarity between these structural changes in the pulmonary arterioles and those which are found in renal arterioles in cases of malignant systemic hypertension. Desclin and Gepts described a similar case in which mitral stenosis was associated with arteriolosclerosis and arteriolitis affecting only the pulmonary vessels; they likened the arteriolitis to polyarteritis nodosa, and suggested that the various vascular changes corresponded to the conditions grouped by Rössle (1933) as the rheumatoid affections of blood vessels.

Only two cases have been reported in which a congenital cardiovascular malformation was associated with a necrotizing arteriopathy limited to the pulmonary arterial tree. In both instances the patient was a boy, aged 11 years, who had been known since birth to have a cardiac lesion which caused exertional dyspnoea and

* A communication read in part before the International Congress of Clinical Pathology in London in July, 1951.

who died after a short, acute illness. In each case the congenital malformation was Eisenmenger's complex. The first case was reported by Old and Russell (1947, 1950); necrotizing arteriopathy, affecting predominantly the smaller muscular arteries but also arterioles, was widespread throughout the lungs and reproduced the histological appearances seen at all stages in the development and healing of the lesions of polyarteritis nodosa, except that eosinophil leucocytes were not found in the inflammatory exudate. The presence of extensive arteriolosclerosis in the lungs was interpreted as indicative of pulmonary hypertension. Old and Russell suggested that pulmonary hypertension was the factor which had determined the exclusively pulmonary distribution of the necrotizing arteriopathy; they did not commit themselves to assigning a cause to the latter. The second case of Eisenmenger's complex with pulmonary arteritis was reported by Kipkie and Johnson (1951); the pathological findings in this case were similar to those described by Old and Russell (1950), but there was in addition acute glomerulonephritis and a history suggesting that the child had been sensitive to sulphonamides, so that the authors considered that a variety of factors might have contributed to the development of the arteritis. They suggested that pulmonary hypertension and other circulatory disturbances resulting from the cardiovascular malformation could have determined the pulmonary localization of the arteritis, and that the latter might have been due either to whatever factor had caused the coexistent nephritis or to allergy to sulphonamides.

McKeown (1952), in a series of 6,770 necropsies, found only four cases in which there was an arteritis limited to the lungs. The patients had presented clinically with right-sided cardiac failure of several months' duration and of obscure origin, and in each case unexplained hypertrophy of the right ventricle was found at necropsy. The vascular lesions were histologically identical with polyarteritis nodosa. McKeown suggested that these were cases of primary arteritis involving only the pulmonary vessels, and that the pulmonary hypertension was secondary to the arteritis and not the cause of it.

From this brief résumé of the literature it can be seen that necrotizing arteriopathy, limited to the pulmonary vasculature, has been observed in cases of pulmonary hypertension secondary to congenital and acquired heart disease and also in cases of pulmonary hypertension in which no other pulmonary or cardiac lesion was demonstrable to account for the hypertension. Some of the authors considered that the necrotizing arteriopathy was partly or wholly allergic in origin, and therefore aetiologically related to polyarteritis nodosa, while others assumed that it was secondary to hypertension. Which of these interpretations was adopted by individual observers seems to have been determined mainly by the supposed morphological resemblance of the lesions in their material to polyarteritis nodosa, suggesting allergy, or to hypertensive systemic arteriolonecrosis.

In both of the following cases certain of the pulmonary vascular changes appeared morphologically to be the counterpart of hyperplastic arteriolosclerosis as seen in the systemic vasculature in essential hypertension; other pulmonary arterioles showed a lesion which appeared to correspond to the arteriolonecrosis seen in systemic arterioles in malignant hypertension, while the remainder of the arteriolar lesions exactly reproduced the picture characteristic of generalized polyarteritis nodosa.

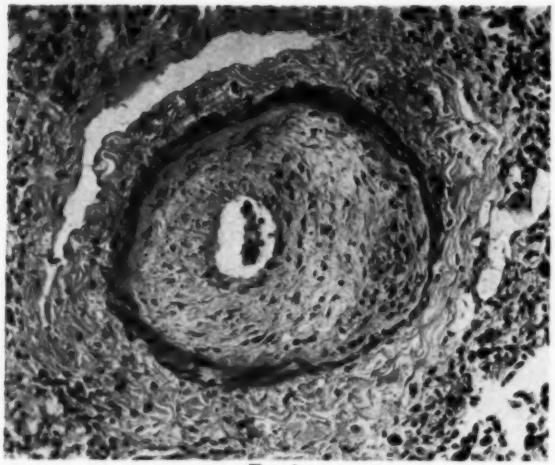


FIG. 1

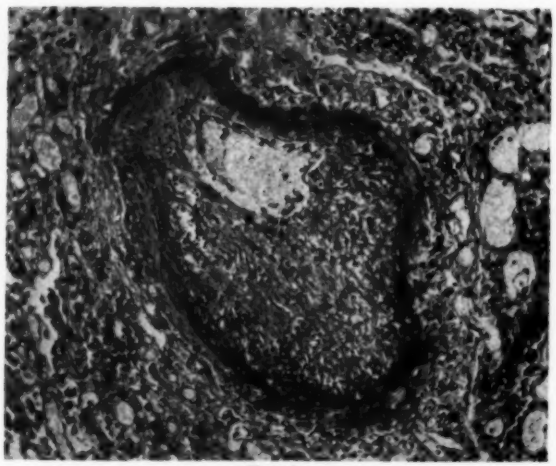


FIG. 2

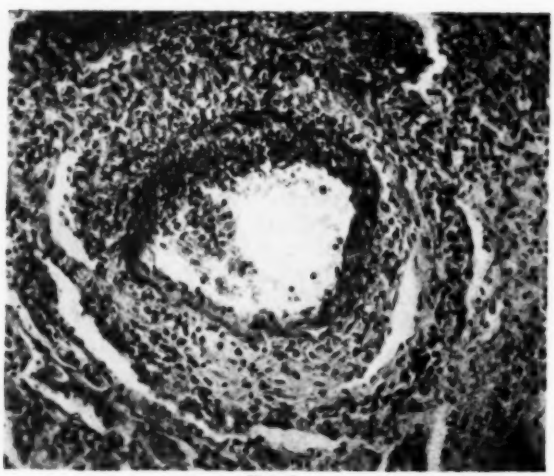


FIG. 3

Report of Cases

Case 1.—A previously healthy man, aged 24, developed a relentlessly progressive, right-sided heart failure of which he died within 18 months of its onset.

Necropsy revealed severe chronic systemic venous engorgement and oedema of cardiogenic distribution. The heart weighed 520 g.; the right ventricle was greatly hypertrophied, whereas the left side of the heart was normal. No valvular lesions and no congenital malformations of the heart or great vessels were found. There was slight fatty atheroma of the larger pulmonary arteries. The lungs were moderately oedematous and engorged, and the branches of the pulmonary arterial tree stood out patulous and thick-walled on the cut surfaces. There was no pulmonary emphysema, fibrosis, or inflammation.

Histological examination showed severe chronic venous congestion of the viscera, but no other vascular abnormality was found except in the lungs. The pulmonary vascular changes were practically uniformly distributed in all parts of the lungs. In the largest pulmonary arteries atheroma and slight pseudocystic mucinoid degeneration of the media were present. There was medial hyperplasia and moderate intimal fibrosis in the small muscular pulmonary arteries, and very marked hyperplastic sclerosis of the pulmonary arterioles (Fig. 1). In addition, there was an extensive necrotizing arteriopathy. Only vessels of the order of 150 μ or less in external diameter showed lesions of the latter variety, and, as far as

FIG. 1.—Case 1: pulmonary arteriolosclerosis. (Haematoxylin and eosin, × 160.)

FIG. 2.—Case 1: pulmonary arteriolosclerosis with fibrinoid necrosis of the media. (Haematoxylin and eosin, × 140.)

FIG. 3.—Case 1: polyarteritis-nodosa-like lesion of a pulmonary vessel, showing segmental distribution of the necrosis and cellular reaction. (Haematoxylin and eosin, × 200.)

could be judged from study of serial sections, this process appeared to be limited to branches of the pulmonary tree, the bronchial vasculature being quite normal. The earliest stage of this necrotizing arteriopathy was the development of fibrinoid necrosis of the muscular coat (Fig. 2), sometimes with small haemorrhages into the fibrinoid material. In later stages, neutrophil polymorphonuclear leucocytes and a smaller proportion of large and small mononuclear inflammatory cells accumulated in all coats of the affected vessel and in the adjacent perivascular tissue; eosinophil leucocytes were very scanty. These more severe changes were often limited to only a segment of the wall of the affected vessel (Fig. 3).

Case 2.—This patient gave a history of recurrent attacks of acute rheumatic fever between the ages of 14 and 20 years, leading to mitral stenosis. His exercise tolerance remained fairly good until the age of 32, when he had an acute febrile illness, shortly after the onset of which a coagulase-positive *Staphylococcus aureus* was isolated from the blood in pure culture on two occasions. The clinical picture was that of septicaemia, but there was nothing otherwise suggestive of a bacterial endocarditis. He recovered from this illness on treatment with penicillin, but his cardiac reserve never regained its previous level. Recurrent episodes of congestive cardiac failure occurred, and during one of these, two years after the septicaemia, he died.

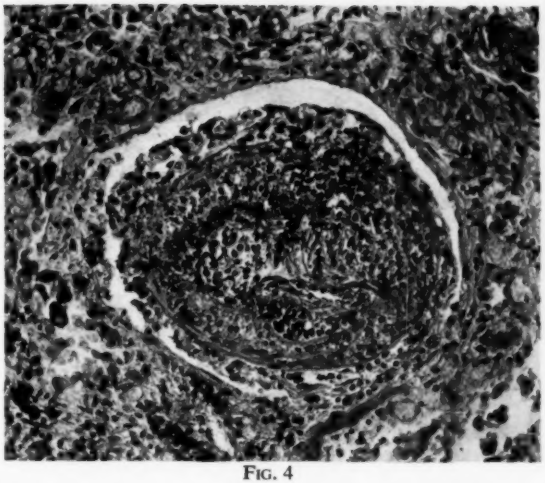


FIG. 4

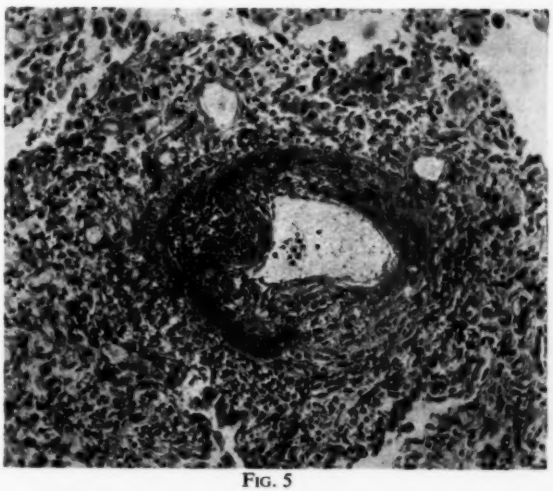


FIG. 5

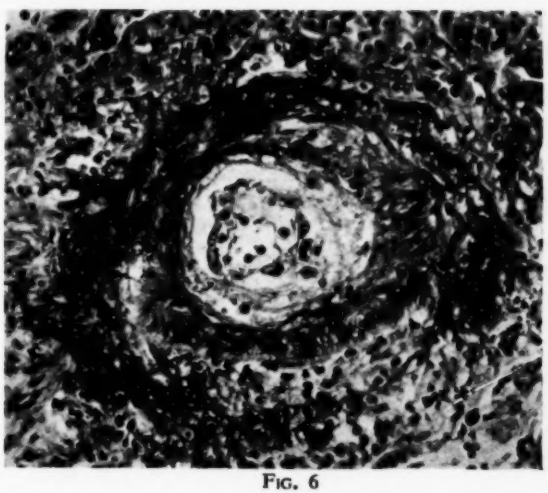


FIG. 6

FIG. 4.—Case 2: pulmonary arteriole showing fibrinoid necrosis in parts of the media and of the thickened intima, with round-cell infiltration. (Haematoxylin and eosin, × 180.)

FIG. 5.—Case 2: polyarteritis-nodosa-like lesion of a pulmonary vessel, showing segmental distribution of the reaction. (Haematoxylin and eosin, × 140.)

FIG. 6.—Case 2: small pulmonary arteriole with fibrinoid necrosis of the media and accumulation of fibrinoid material in the perivascular tissue. (Haematoxylin and eosin, × 290.)

Necropsy showed calcification and extreme stenosis of the mitral valve, and marked hypertrophy of the right ventricle which accounted for most of the cardiac enlargement (heart weight 610 g.). The other findings were essentially the same as in Case 1.

The histological findings (Figs. 4 and 5) in the lungs were similar to those in Case 1, except that a few of the smallest calibre arterioles showed a considerable accumulation of fibrinoid material in the perivascular tissue in addition to fibrinoid necrosis of the media (Fig. 6).

Discussion

The pathological findings in Case 1 were not considered to be of such a nature as could explain the cause of the pulmonary hypertension, and the case was therefore relegated to the unsatisfactory category of essential pulmonary hypertension. In Case 2, on the other hand, it was apparent that right ventricular hypertrophy and failure had developed essentially as a consequence of mitral stenosis and the resulting impediment in the lesser circulation.

In each case the necrotizing arteriopathy was thought to be pathogenetically akin to the arteriolar necrosis of malignant systemic hypertension. Although the histological appearances in certain vessels were indistinguishable from those seen in cases of generalized polyarteritis nodosa, the lesions in the majority of the arterioles far more closely resembled the picture of hypertensive systemic arteriolonecrosis, with fibrinoid necrosis of the tunica media and very little or no cellular reaction or other evidence of inflammation. It was felt that the polyarteritis-nodosa-like lesions had developed as an inflammatory response secondary to the vasonecrosis, and there did not seem to be any grounds for suggesting that allergy was a factor in their genesis. There was no history of any allergic state in Case 1, and the only possibly allergic disease in Case 2 was the recurrent rheumatic fever during the patient's youth. No evidence of any active rheumatic condition was found at necropsy in either case, and the pulmonary vascular lesions did not closely resemble rheumatic angiitis. Penicillin and sulphonamides had been used on various occasions in the treatment of each patient, but there was never any clinical evidence to suggest that either patient had developed hypersensitivity to these drugs.

It is not possible to do more than speculate about the precise mechanism by which the necrotizing and inflammatory vascular changes developed. Factors such as local oxygen deficiency and vasospasm may have interacted with hypertensive mechanical stresses to initiate the necrosis. There can be no doubt that the necrotizing arteriopathy was of considerably shorter duration than the clinical history of right-sided cardiac failure, and that it was not the cause of the latter. The absence of any unequivocal evidence of healing in the affected vessels suggested, in fact, that the necrotizing arteriopathy was of fairly recent onset, and it is noteworthy that in each case there had been a comparatively sudden and rapidly progressive increase in the severity of the cardiac failure in the period of two to three months preceding the patient's death.

It has already been mentioned that in the literature the necrotizing arteriopathy in the lungs of patients with pulmonary hypertension has been described by some authors as polyarteritis nodosa and by others as morphologically similar to hypertensive systemic arteriolonecrosis. The presence of both hypertensive arteriolonecrotic and polyarteritis-nodosa-like lesions in the two cases reported here is therefore of particular interest. As the resemblance to polyarteritis nodosa has

been considered by other authors to indicate that such vascular lesions in cases of pulmonary hypertension are allergic in nature, it is relevant in conclusion to refer to the hypertensive hypothesis of the aetiology of polyarteritis nodosa. Smith and Zeek (1947) opposed the view that polyarteritis nodosa is an allergic disease ; they suggested instead that arterial hypertension plays the main role in its pathogenesis. Their evidence to support this suggestion was that lesions closely resembling if not identical with those of polyarteritis nodosa develop in a proportion of animals in which severe hypertension has been rapidly produced by any of a variety of experimental procedures. Zeek, Smith, and Weeter (1948) further claimed that polyarteritis nodosa, being of hypertensive origin, can be distinguished clinically and pathologically from another variety of necrotizing panarteritis which they believed to be genuinely of allergic origin, and which they named " hypersensitivity angiitis." This is not the place to attempt a detailed criticism of these views, but, although the findings in cases of severe pulmonary hypertension, such as the above, show that a polyarteritis-like arteriopathy can occur in vessels exposed to hypertension, there is much evidence against the general applicability of the hypertensive hypothesis of the nature of polyarteritis nodosa. The reaction of the pulmonary vasculature in some cases of pulmonary hypertension is nevertheless of interest in relation to the problem of generalized polyarteritis nodosa, and may repay further study.

Summary

Two cases are described in which there was a necrotizing arteriopathy limited to the pulmonary vasculature. Essential (primary) pulmonary hypertension was present in one case, and pulmonary hypertension secondary to mitral stenosis in the other. The lesions in the small blood vessels in each case were of three kinds: (1) arteriolosclerosis, comparable to hypertensive systemic arteriolosclerosis ; (2) fibrinoid necrosis of the media of small arteries and arterioles, comparable to the arteriolonecrosis associated with malignant systemic hypertension ; and (3) arteritis and arteriolitis, reproducing the histological features seen in polyarteritis nodosa.

The relevant literature is briefly reviewed, with particular reference to the various interpretations which have been suggested in explanation of these pulmonary arterial changes in cases of pulmonary hypertension.

It is suggested that necrotizing arteriopathy limited to the pulmonary vasculature and occurring in association with pulmonary hypertension is a result of the hypertension and not its cause.

I should like to thank Dr. E. Florence McKeown, of the Institute of Pathology, Queen's University of Belfast, for allowing me access to her study of pulmonary heart disease before its publication.

REFERENCES

Desclin, L., and Gepts, W. *Proc. int. Congr. clin. Path.*, London. To be published.
Kipkie, G. F., and Johnson, D. S. (1951). *Arch. Path., Chicago*, **51**, 387.
McKeown, F. (1952). *Brit. Heart J.*, **14**, 25.
Old, J. W., and Russell, W. O. (1947). *Amer. J. Path.*, **23**, 903.
—— —— (1950). Ibid., **26**, 789.
Parker, F., jun., and Weiss, S. (1936). Ibid., **12**, 573.
Rössle, R. (1933). *Virchows Arch.*, **288**, 780.
Smith, C. C., and Zeek, P. M. (1947). *Amer. J. Path.*, **23**, 147.
Zeek, P. M., Smith, C. C., and Weeter, J. C. (1948). Ibid., **24**, 889.

J. clin. Path. (1952), **5**, 42.

LIVER GLYCOGEN IN DIABETES MELLITUS

BY

J. VALLANCE-OWEN

From The London Hospital

(RECEIVED FOR PUBLICATION JULY 20, 1951)

The present consensus of opinion regarding the amount of liver glycogen in diabetes mellitus is well exemplified by Korenberg (1943), "Although opinions differ as to the exact mechanism responsible for the syndrome of diabetes mellitus, unanimity exists with regard to the fact that in that condition the hepatic glycogen is decreased . . ." and Mirsky (1942), "The most important cause of diabetic ketosis and hence of the subsequent acidosis and coma is the impoverishment of the liver with respect to glycogen."

These statements are based largely on experimental work with depancreatized animals following the discovery of von Mering and Minkowski (1890) that extirpation of the pancreas in dogs resulted in permanent diabetes mellitus corresponding to the most severe form of the disease in man. These workers found only traces of glycogen in the livers of these animals and this observation has been confirmed repeatedly (Cruickshank, 1913; Banting, Best, Collip, Macleod, and Noble, 1922; Hédon, 1925; Fisher and Lackey, 1925; Major and Mann, 1932; and Bodo, Co Tui, and Farber, 1933).

More recently, experiments with alloxan-diabetic animals have agreed closely with the above finding. Although the liver-glycogen stores may be normal in the less severe forms of alloxan diabetes, the amount appears invariably to be reduced to minute quantities when ketosis and coma supervene, as in depancreatized animals (Goldner and Gomori, 1943; Hard and Carr, 1944; Kaplan, Franks, and Friedgood, 1945; Weber, 1946; Tuerkischer and Wertheimer, 1946 and 1947). The variability observed is possibly due to the continued production of insulin in the less severe forms, as the β-cells of the islet tissue in these animals may not have been completely necrosed.

My interest in this subject was aroused by a young diabetic, aged 21, who died in severe ketosis and coma one and a half hours after an injection of 80 units of soluble insulin. Here the glycogen content of the liver, as demonstrated histologically by Best's carmine after fixation in Bouin's fluid seven hours after death, was extremely high, the picture resembling that seen in von Gierke's disease. It seems unlikely that the deposition of glycogen could have occurred solely from the action of exogenous insulin in the time available and more probable that normal quantities of glycogen were present before the injection was given. But before making further observations on the glycogen content of the liver in human necropsy material it was obviously necessary to re-assess the rapidity with which glycogen disappears from the liver cells after death, and thus the value in general of such material.

Although glycogen is believed to disappear rapidly from the tissues after death, Kütz (1880–1) was able to estimate considerable quantities of glycogen in samples of unfixed livers of various animals after keeping the tissues at room temperature for as long as eight days. It appears from this early observation and from later work (Garnier and Lambert, 1897; Bobbitt and Deuel, 1940; Swensson, 1945) that, although glycogen does disappear from the tissues after death, the disappearance is far slower than is generally supposed. Moreover, special fixation of the tissues in alcohol or Bouin's fluid is not essential for the demonstration of glycogen.

In a preliminary investigation using rabbits' livers (Vallance-Owen, 1948) it was shown that formaldehyde is as efficient as either of the two fixatives mentioned; also there is no appreciable loss of stainable glycogen, either during storage of liver tissue at a temperature of about 3° C. for periods up to 48 hours, or by the subsequent processing of the tissue in the same manner as for human necropsy material. Therefore, even if quantitative estimations were to show some initial loss of glycogen within the first few minutes after death, it is permissible to use formalin-fixed necropsy material for the histological demonstration of glycogen and to compare different series of cases in parallel. Moreover, the histological approach is strengthened by the findings of Popper and Wozasek (1931), who showed that there is a close similarity between the amounts so demonstrated and the actual quantities found on biochemical estimation.

Present Investigation

The series examined comprised 31 patients with diabetes mellitus dying in diabetic coma and 25 controls. Sections of the livers, formalin-fixed and paraffin-blocked, from the diabetic and control series, of an average thickness of 6–7 μ, were floated on 70% alcohol at 55–60° C., mounted and dried, and after the paraffin had been removed were covered with a film of 1% celloidin. They were then stained with haematoxylin and Best's carmine (Carleton and Leach, 1938). Control sections in each case were treated with saliva before staining.

On microscopic examination the sections were graded in respect of the amount of glycogen present in the cytoplasm of the liver cells, according to the following scheme: + indicated several lobules devoid of glycogen, elsewhere granules scattered diffusely through the liver lobule or in well-circumscribed areas; ++ small glycogen particles scattered throughout almost the whole of each lobule with or without increased deposition in certain areas; +++ glycogen particles larger and slightly more numerous than in ++; ++++ glycogen particles further increased in number and size; +++++ glycogen in large quantities throughout the liver parenchyma. This is only to give an idea of how the quantity of glycogen was assessed, the individual variation in the distribution being recorded separately.

Diabetic Series.—The cases were divided into those which had not received insulin, 15 cases, of which 12 dated to the years before insulin was introduced, and those which had received insulin, 16 cases. All these patients had gross glycosuria and ketonuria and died in coma (see Appendix).

Control Series.—This consists of 25 cases dying of conditions other than diabetes mellitus, in which carbohydrate metabolism was not involved and where the liver was not diseased. Thirteen of these cases were chronic hospital patients whose death occurred from debilitating diseases. The remaining 12 patients died rapidly from acute medical conditions or as a result of accidents. The latter are obviously preferable as "normal" controls, but the former group is included, since Popper and

Wozasek (1931) argued that it was more reasonable to compare the livers of diabetics with those of patients dying from some other cachectic state.

Both in the diabetic and control series all the livers in which glycogen could be demonstrated were found to be devoid of it following treatment with saliva.

Results

From Table I it can be seen that the livers in the control series where death was due to a debilitating disease had an uneven content of glycogen. In general it was

TABLE I

RESULTS OF ANALYSES FOR GLYCOGEN IN DIABETIC AND CONTROL LIVERS

Diabetic Series				Control Series			
Case No.	No Insulin	Case No.	Insulin	Case No.	Debilitating Conditions	Case No.	Sudden Death (" Normal ")
1	Nil	16	+++	1	Nil	14	++
2	+++	17	++	2	Nil	15	++
3	++	18	++++	3	++	16	+++
4	++	19	Nil	4	Nil	17	+++
5	Nil	20	Nil	5	+	18	++
6	+++	21	++	6	Nil	19	+
7	Nil	22	++	7	+	20	++
8	+	23	Nil	8	++	21	++
9	++++	24	++	9	+	22	++
10	++	25	+++	10	Nil	23	+++
11	Nil	26	+++	11	+	24	+++
12	++++	27	+++++	12	Nil	25	+++
13	Nil	28	++++	13	Nil		
14	+++	29	++++				
15	+++	30	+++				
		31	+++++				

small and frequently it was absent. In the patients dying rapidly from acute medical conditions or as a result of accidents the livers were reasonably uniform and contained much more glycogen ; these have been taken as normal controls. Although six of the 15 livers of patients dying in diabetic ketosis and coma without insulin showed little or no glycogen, the remaining nine showed as much or more than the normal controls and considerably more than in those of patients dying from debilitating diseases. The diabetics dying in coma, having received insulin, showed a uniformly increased deposition of glycogen in the liver over the controls if the cases before 1931 are excluded (Cases 16–24). Where glycogen was present, it was most marked in the cells of the periportal region, much less, and frequently none, being demonstrated round the central vein, although present elsewhere throughout the lobule. There appeared to be no antagonism between the deposition of glycogen and fat ; both were often present in large amounts.

Discussion

These findings agree well with the early observations of Külz (1880–1), Geelmuyden (1920), Warren (1930), and Popper and Wozasek (1931). All these workers found normal or increased amounts of liver glycogen in many of their patients dying in diabetic coma, although they had received no insulin.

Recently Hildes, Sherlock, and Walshe (1949) applied the aspiration biopsy technique for obtaining specimens of liver to unanaesthetized diabetic patients. They were unable to obtain a statistically significant difference between the values for liver glycogen in their diabetic and control series. But though insulin was discontinued at least two days before the biopsies were taken, the majority of the cases were only mildly ketotic. Using the same technique, Bondy, Sheldon, and Evans (1949) found reduced quantities of glycogen in the majority of their cases with diabetic ketosis, but they had one patient in semicoma from that condition in whom greatly increased quantities were found. The results are at variance with those in experimental depancreatized and alloxan-diabetic animals where hepatic glycogen deposits are uniformly reduced to minimal traces when ketosis occurs.

Turning to the cases of the present series which had been treated with insulin, the results from 1931 onwards (Cases 25–31) have been remarkably uniform and show an increased deposition of glycogen. This date coincides with the advent of pure crystalline insulin. Previously, impure, non-crystalline insulin was used and this apparently contains varying amounts of a glycogenolytic factor, as has been pointed out by Bouckaert and de Duve (1947), and has been responsible for the early conflicting reports on the effect of the hormone. The erratic results from 1925 to 1931 in the insulin-treated group can possibly be explained on these grounds. Otherwise the findings here confirm those of the above authors, who are satisfied that, provided the blood sugar is maintained at normal or elevated levels, then hepatic glycogen will regularly be found to increase under the action of pure insulin. They go on to stress that the normal regulator of sugar metabolism is the blood sugar itself and that the effect of insulin depends on it. Also Soskin (1930) showed that every criterion of carbohydrate utilization can be obtained without insulin in the depancreatized animal. Moreover, Mirsky and his colleagues (Mirsky, Heiman, and Broh-Kahn, 1937a, and Mirsky, Heiman, and Swadesh, 1937b) have shown that the antiketogenic and nitrogen-sparing effects of carbohydrate can be obtained in depancreatized dogs, provided the blood sugar is raised to sufficiently high levels. Major and Mann (1932) and Bodo *et al.* (1933) found deposition of glycogen in the livers of their depancreatized animals when given glucose intravenously, although at a rate far below that in the normal animal. Thus it would appear that insulin acts as a catalyst in carbohydrate metabolism, allowing the tissues to achieve at normal or low blood sugar levels that for which high levels are required in its absence.

From the results of other investigators and those presented in this paper, it is clear that cases of human diabetes mellitus in severe ketosis can be divided into two broad groups, those with little or no glycogen in the liver and those with normal or increased amounts of that substance. In view of what has already been stated regarding insulin action and the fact that depancreatized animals invariably show very low levels of hepatic glycogen, the difference in the two groups may be explained on a basis of circulating insulin. Those cases with negligible quantities of glycogen had little or no insulin circulating and the blood sugar had not risen to sufficiently high levels to promote any storage, whereas those with normal or increased amounts of glycogen presumably had appreciable quantities of circulating hormone, perhaps in normal or even increased amounts in some cases, or alternatively had excessively high blood sugar levels. That such high blood sugar

levels alone could cause the elevation of hepatic glycogen storage to even normal amounts has been dismissed as improbable in view of the work of Bodo *et al.* (1933).

As ketosis and coma can occur in diabetes mellitus with normal or increased amounts of glycogen in the liver, the present conception of its aetiology must be revised at least in relation to this disease. For many years the fundamental abnormality in diabetes mellitus was thought to be the non-utilization of sugar. There is now considerable evidence to indicate that the diabetic organism, maintaining a high blood sugar level, is using as much and often more sugar than the normal organism at its usual blood sugar level (Mann and Magath, 1923 ; Macleod and Markowitz, 1926 ; Soskin, 1930 ; Soskin and Levine, 1937 ; Zilversmit, Chaikoff, Feller, and Masoro, 1948). In the light of this work an alternative theory of the mechanism of the diabetic state has been put forward, ascribing the hyperglycaemia and glycosuria to an over-production of glucose from protein and fat, probably in the liver. This excessive production is sufficient to cause accumulation and excretion of excess sugar in spite of the continued oxidation of glucose at the normal rate. Fats break down into glycerol and fatty acid, glycerol is built up to glucose via phosphoglyceraldehyde, and the fatty acids are converted into aceto-acetic acid by way of acetic acid. There is still much doubt as to whether fatty acids can be converted into glucose. But Weil-Malherbe (1938) has demonstrated, in kidney slices from the rat, the *in vitro* formation of sugar from added aceto-acetic acid.

On the basis of the over-production theory of diabetes mellitus the appearance of ketosis in this condition with glycogen-loaded livers can be explained. As the breakdown of fat proceeds, aceto-acetic acid is formed at a rate greater than that of its utilization and possible conversion to sugar, and so ketosis develops. There is no indication that the peripheral tissues of the diabetic organism dispose of ketones any less rapidly than those of the normal animal (Chaikoff and Soskin, 1928). The antiketogenic properties of glucose and insulin in diabetes mellitus would therefore appear to depend on the inhibition of gluconeo-genesis from fat, the former indirectly by raising the blood sugar level artificially (Mirsky *et al.*, 1937a) and the latter directly, and not, as was previously believed, by restoration of liver glycogen.

Excessive gluconeo-genesis would certainly appear to be the mechanism of the production of the diabetic state associated with acromegaly, Cushing's basophilism, and certain tumours of the adrenal cortex (Sprague, Priestley, and Dockerty, 1943). In view of this theory it would seem reasonable to suppose that many cases of diabetes mellitus arise from over-action of the pituitary gland, either directly or through the adrenal. There is, however, no factual evidence to support it, but the overall evidence upon which the pancreas is incriminated is slight. It is well known that, at necropsy, this gland gives no constant indication of the severity of the condition and is frequently normal macroscopically and microscopically (e.g. Warren and Root, 1925 ; Wright, 1927 ; White, 1939 ; Susman, 1942).

It is interesting to note the relationship of islet change to hyperglycaemia studied by Lukens, Dohan, and Wolcott (1943). These workers showed that neither partial pancreatectomy nor injections of anterior pituitary extract would cause hydropic degeneration in the β-cells of the islets in the absence of hyperglycaemia, but both procedures caused it after hyperglycaemia had been present for two to three weeks.

They go on to point out that hyperglycaemia caused by continuous perfusion of glucose can result in minor changes in the islet tissue. From these experiments and observations it seems possible that any physiological or morbid anatomical changes in the pancreas, found occasionally in diabetes mellitus, are merely secondary to the hyperglycaemia produced by excessive gluconeo-genesis through some agent, possibly the pituitary gland, as yet unknown. As might be expected, any changes found seem to be confined to the older patients, in whom the islet tissue is ageing and vascular factors come into play.

Finally, completely depancreatized human beings can be kept quite stable on small quantities of insulin, such as 20 units twice a day, as was shown by Goldner and Clarke (1944), and now the phenomenon is seen frequently in patients who have undergone pancreatectomy for carcinoma of the pancreas. This is a strong argument for believing that the picture in depancreatized and alloxan-diabetic animals is not truly comparable with diabetes mellitus as seen in man, and readily explains the differences in the literature which have occurred when comparing these two groups, particularly with reference to liver glycogen. It must now be believed that in many cases of diabetes mellitus normal quantities of insulin are circulating and that the condition is due, not to some pathological condition of the pancreas, but rather to some other cause or causes which have not yet been firmly established.

Summary

The liver glycogen has been studied histologically in 31 patients with diabetes mellitus dying in coma and compared with controls.

It has been shown that diabetic ketosis and coma can occur with normal or increased glycogen deposits in the liver. Such normal or increased glycogen deposits are frequently found in untreated human diabetes mellitus. Pure insulin causes an increase in glycogen deposition in the liver.

The bearing of these findings upon present conceptions of diabetes mellitus is discussed.

I should like to thank Professor Dorothy Russell for her invaluable encouragement and criticism, and Professor J. R. Marrack for assistance with the biochemistry.

At one stage during the completion of this work I was in receipt of a grant from the Freedom Research Fund of The London Hospital, for which I am much indebted.

BIBLIOGRAPHY

Banting, F. G., Best, C. H., Collip, J. B., Macleod, J. J. R., and Noble, E. C. (1922). *Trans. roy. Soc. Can.*, **16**, Sect. V, 39.
Bobbitt, B. G., and Deuel, H. J. (1940). *Amer. J. Physiol.*, **131**, 521.
Bodo, R. C., Co Tui, F., and Farber, L. (1933). Ibid., **103**, 18.
Bondy, P. K., Sheldon, W. H., and Evans, L. D. (1949). *J. clin. Invest.*, **28**, 1216.
Bouckaert, J. P., and de Duve, C. (1947). *Physiol. Rev.*, **27**, 39.
Carleton, H. M., and Leach, E. H. (1938). *Histological Technique*, 2nd ed., pp. 106 and 171. London.
Chaikoff, I. L., and Soskin, S. (1928). *Amer. J. Physiol.*, **87**, 58.
Cruickshank, E. W. H. (1913). *J. Physiol., Lond.*, **47**, 1.
Fisher, N. F., and Lackey, R. W. (1925). *Amer. J. Physiol.*, **72**, 43.
Garnier, L., and Lambert, M. (1897). *C. R. Soc. Biol., Paris*, 10 ser, **4**, 718.
Geelmuyden, H. C. (1920). *Acta med. scand.*, **53**, 381.
Goldner, M. G., and Clark, D. E. (1944). *J. clin. Endocrinol.*, **4**, 194.
—— and Gomori, G. (1943). *Endocrinology*, **33**, 297.
Hard, W. L., and Carr, C. J. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 214.
Hédon, E. (1925). *C. R. Soc. Biol., Paris*, **93**, 596.

Hildes, J. A., Sherlock, S., and Walshe, V. (1949). *Clin. Sci.*, **7**, 287.
Kaplan, N. O., Franks, M., and Friedgood, C. E. (1945). *Science*, **102**, 447.
Korenberg, M. (1943). *Arch. intern. Med.*, **72**, 746.
Külz, E. (1880–1). *Pflüg. Arch. ges. Physiol.*, **24**, pp. 1–114.
Lukens, F. D. W., Dohan, F. C., and Wolcott, M. W. (1943). *Endocrinology*, **32**, 475.
Macleod, J. J. R., and Markowitz, J. (1926). *Trans. Ass. Amer. Phys.*, **41**, 147.
Major, S. G., and Mann, F. C. (1932). *Amer. J. Physiol.*, **102**, 409.
Mann, F. C., and Magath, T. B. (1923). *Arch. intern. Med.*, **31**, 797.
Mering, J. von, and Minkowski, O. (1890). *Arch. exp. Path. Pharmak.*, **26**, 371.
Mirsky, I. A. (1942). *J. Amer. med. Ass.*, **118**, 690.
—— Heiman, J. D., and Broh-Kahn, R. H. (1937a). *Amer. J. Physiol.*, **118**, 290.
—— —— and Swadesh, S. (1937b). Ibid., **120**, 681.
Popper, H., and Wozasek, O. (1931). *Virchows Arch.*, **279**, 819.
Soskin, S. (1930). *J. Nutrit.*, **3**, 99.
—— and Levine, R. (1937). *Amer. J. Physiol.*, **120**, 761.
Sprague, R. G., Priestley, J. T., and Dockerty, M. B. (1943). *J. clin. Endocrinol.*, **3**, 28.
Susman, W. (1942). Ibid., **2**, 97.
Swensson, A. (1945). *Acta physiol. scand.*, Suppl. 33, p. 47.
Tuerkischer, E., and Wertheimer, E. (1946). *Nature, Lond.*, **158**, 201.
—— —— (1947). *J. Endocrinol.*, **5**, 229.
Vallance-Owen, J. (1948). *J. Path. Bact.*, **60**, 325.
Warren, S. (1930). *Amer. J. med. Sci.*, **179**, 482.
—— and Root, H. F. (1925). *Amer. J. Path.*, **1**, 415.
Weber, H. (1946). *Nature, Lond.*, **158**, 627.
Weil-Malherbe, H. (1938). *Biochem. J.*, **32**, 2276.
White, P. (1939). *Arch. intern. Med.*, **63**, 39.
Wright, A. W. (1927). *Amer. J. Path.*, **3**, 461.
Zilversmit, D. B., Chaikoff, I. V., Feller, D. D., and Masoro, E. J. (1948). *J. biol. Chem.*, **176**, 389.

CLINICAL APPENDIX

Diabetic Series

The following cases did not receive insulin.

Case 1.—Male, 65 years. Carbuncle of neck precipitated the coma ; duration not known.

P.M., 232/11, 48 hours after death. Body weight 140 lb. Liver (3 lb. 14 oz.) congested and fatty. Microscopically moderate fatty infiltration. Glycogen absent.

Case 2.—Female, 24 years. Diabetes for five years, on diet. Coma five days after admission and died after further two days.

P.M., 675/12, less than 24 hours after death. Body weight 75 lb. Liver (?) oedematous and engorged, with, microscopically, moderate fatty infiltration. Glycogen +++, diffuse with accentuation round portal systems and central veins.

Case 3.—Male, 14 years. Ill for 14 days with cold ; developed wasting, great thirst and polyuria ; one day into coma ; died four and a half hours after admission.

P.M., 134/15, less than 24 hours after death. Body weight 58 lb. Liver (1 lb. 14 oz.) showed fatty degeneration with few submiliary granulomatous tubercles in capsule, and microscopically gross fatty degeneration. Glycogen ++, prominent round portal veins and midzones, absent round central veins.

Case 4.—Male, 55 years. One year diabetic symptoms, on diet. Two weeks influenza. Three days in coma and two days before death blood sugar 0.6%.

P.M., 632/20, 24 hours after death. Body weight 87 lb. Liver (2 lb. 8 oz.) showed brown atrophy and microscopic-

ally slight to moderate fatty degeneration. Glycogen + +, prominent round portal systems extending to midzones, absent round central veins.

Case 5.—Female, 35 years. Six months symptoms of diabetes mellitus. In coma one day before admission, died in 12 hours.

P.M., 190/21, 30 hours after death. Body weight 95 lb. Liver (3 lb. 4 oz.) showed oedema and cloudy swelling. Microscopically fatty degeneration slight. Glycogen almost absent, but traces around portal systems.

Case 6.—Male, 36 years. Six months diabetic symptoms. In coma two days, blood sugar 0.44% one day before death.

P.M., 578/21, 24 hours after death. Body weight 89 lb. Liver (3 lb. 10 oz.) congested with fatty degeneration. Microscopically fatty degeneration moderate to severe. Glycogen + + +, small quantities scattered throughout the lobules but main component in well circumscribed areas in varying parts of the liver lobule.

Case 7.—Male, 21 years. Seven days "acute diabetes mellitus," died in coma three days after admission.

P.M., 232/22, 106 hours after death. Body weight 106 lb. Liver (3 lb. 5 oz.) showed congestion and moderate parenchymatous degeneration with scattered pinhead focal necrosis. Microscopically fatty change slight. Glycogen was absent.

Case 8.—Female, 38 years. Into coma one day, admitted and died in 12 hours.

P.M., 315/22, 30 hours after death. Body weight 99 lb. Liver (5 lb. 12 oz.) showed parenchymatous degeneration. Microscopically fatty degeneration slight. Glycogen +, periportal distribution.

Case 9.—Female, 46 years. Five years increasing thirst and appetite with developing acromegaly. Admitted in coma and died in two days.

P.M., 435/22, 20 hours after death. Body weight 145 lb. Liver (7 lb. 3 oz.) enlarged and flabby with scattered areas of focal necrosis. Microscopically fatty degeneration moderate. Glycogen + + + +, diffuse with slight accentuation round portal systems.

Case 10.—Female, 70 years. Gangrene left foot precipitated ketosis. Blood sugar one week before death 0.23%, in coma two days.

P.M., 468/22, 24 hours after death. Body weight 143 lb. Liver (3 lb. 12 oz.) showed fine reticular fibrosis. Microscopically fatty degeneration slight, fibrosis marked. Glycogen + +, scattered diffusely through the lobule with slight accentuation round portal systems.

Case 11.—Female, 67 years. Three years diabetes mellitus, gangrene right foot precipitated coma. Blood sugar three days before death 0.3%.

P.M., 480/22, 20 hours after death. Body weight 157 lb. Liver (3 lb. 13 oz.) showed fatty degeneration, and microscopically marked fatty degeneration. Glycogen was absent.

Case 12.—Female, 50 years. Ten days unwell with headache. Seven days discharging right ear with increasing appetite, polyuria, and polydipsia. Four days, semicoma, fluctuating in depth. One day, deep coma.

P.M., 519/22, 28 hours after death. Body weight 176 lb. Liver (4 lb. 6 oz.) large and flabby with fatty degeneration. Microscopically moderate fatty degeneration. Glycogen + + + +, diffuse with no particular accentuation.

Case 13.—Female, 73 years. Diabetes mellitus for many years, on 30 units b.d. Admitted in coma and died quickly. No insulin given.

P.M., 198/33, 12 hours after death. Body weight 327 lb. Liver (3 lb. 12 oz.) showed central congestion and atrophy. Microscopically fatty degeneration moderate. Glycogen was absent.

Case 14.—Female, 74 years. Seventeen years diabetes mellitus, no insulin. Admitted in semicoma, blood sugar 0.29%. Passed into deep coma and no insulin given.

P.M., 403/34, six hours after death. Body weight 173 lb. Liver (3 lb.) oedematous. Microscopically fatty degeneration moderate. Glycogen + + +, diffuse with accentuation round portal systems.

Case 15.—Female, 67 years. Fourteen years diabetes mellitus, on soluble insulin 20 : 40. One day, drowsy with occasional epileptic fits. Blood sugar 0.24% on admission. Fits controlled by potassium bromide and chloral, but given no insulin. Gradually into deepening coma and died two days later.

P.M., 394/38, four hours after death. Body weight 87 lb. Liver (2 lb. 9 oz.) showed diffuse congestion. Microscopically fatty degeneration moderate. Glycogen + + +, diffuse with slight accentuation round portal systems.

The following cases received insulin.

Case 16.—Male, 21 years. Three weeks symptoms of diabetes mellitus, admitted in semicoma. Insulin 40 units three-hourly and blood sugar down to 0.12%. Last insulin injection 14 hours before death, and three hours before death blood sugar 0.5%.

P.M., 83/25, 40 hours after death. Body weight 105 lb. Liver (3 lb. 4 oz.) showed oedema, congestion, and fatty degeneration. Microscopically fatty degeneration moderate. Glycogen + + +, diffuse with slight accentuation round portal systems.

Case 17.—Female, 42 years. Four years diabetes mellitus, admitted in coma, given 60 units insulin, died "in a few hours."

P.M., 88/27, 36 hours after death. Body weight 86 lb. Liver (2 lb. 11 oz.) showed focal necrosis. Microscopically fatty degeneration slight. Glycogen + +, diffuse with slight accentuation round portal systems.

Case 18.—Female, 47 years. No previous diabetic history, admitted in semicoma. Blood sugar not recorded. Given 30 units soluble insulin on admission and thereafter 20 units two-hourly for 48 hours, i.e., 510 units in all, but died in coma 72 hours after admission.

P.M., 96/27, 22 hours after death. Body weight 131 lb. Liver (3 lb. 3 oz.) showed oedema and parenchymatous degeneration. Microscopically fatty degeneration moderate. Glycogen + + + +, diffuse.

Case 19.—Female, 16 years. Three years diabetic, admitted in coma. Blood sugar 0.44%. Given 95 units insulin at once but no effect, died five hours after admission.

P.M., 392/27, 28 hours after death. Body weight 118 lb. Liver (3 lb. 7 oz.) showed oedema and severe parenchymatous degeneration. Microscopically fatty degeneration slight. No glycogen.

Case 20.—Female, 30 years. One year diabetes mellitus, on diet and 25 units insulin b.d. Admitted in coma, blood sugar 0.4%, insulin 50 units hourly for six hours. Blood sugar then 0.33%, died in coma seven hours after admission.

P.M., 572/27, 11 hours after death. Body weight 93 lb. Liver (3 lb. 4 oz.) showed oedema, congestion, and parenchymatous degeneration. Microscopically fatty degeneration moderate. No glycogen.

Case 21.—Female, 64 years. Three days before became "ill and weak." Admitted in coma, blood sugar 0.4%, given 50 units insulin hourly for seven hours, last injection three hours before death. One hour before death blood sugar 0.42%.

P.M., 14/28, nine hours after death. Body weight 85 lb. Liver (2 lb. 12 oz.) showed oedema and central congestion. Microscopically fatty degeneration very slight. Glycogen + +, irregular with greatest accumulation round portal systems.

Case 22.—Female, 46 years. One week carbuncle of neck, two weeks frequency and thirst. Admitted in coma. Blood sugar 0.4%. Given insulin 30 units hourly for three hours, no effect. Died four hours after admission.

P.M., 325/28, 20 hours after death. Body weight 99 lb. Liver (3 lb. 11 oz.) showed post-mortem degeneration. Microscopically fatty degeneration, slight to moderate. Glycogen + +, diffuse.

Case 23.—Female, 19 years. Four days ill, weak and drowsy. Admitted in coma, blood sugar 0.65%. Given 50 units insulin intravenously, died in one and a half hours.

P.M., 416/29, 39 hours after death. Body weight 91 lb. Liver (2 lb. 7 oz.) showed oedema, congestion, and parenchymatous degeneration. Microscopically fatty change moderate. No glycogen.

Case 24.—Male, 19 years. Sore throat and thirst, admitted in coma. Blood sugar 1.0%, given 100 units insulin at once and 50 units one hour later. Died three hours after admission.

P.M., 298/30, 40 hours after death. Body weight 94 lb. Liver (2 lb. 14 oz.) showed severe parenchymatous degeneration. Microscopically fatty degeneration severe. Glycogen + +, diffuse.

Case 25.—Male, 30 years. Diabetic on 10 units insulin b.d. Had diabetic cataract. Admitted in coma. Blood sugar 0.8%. Insulin 50 units. Died three-quarters of an hour after admission.

P.M., 96/31, 12 hours after death. Body weight 166 lb. Liver (3 lb. 9 oz.) showed oedema, congestion, and focal degeneration. Microscopically fatty change severe. Glycogen + + +, diffuse but irregular with accentuation round portal systems.

Case 26.—Female, 25 years. One year diabetes mellitus, on diet, and 40 units t.d.s. Admitted in coma. Blood sugar 0.28%. Given 30 units insulin at once and four hours later 40 units. Died eight hours after admission.

P.M., 151/31, 13 hours after death. Body weight 120 lb. Liver (5 lb.) showed severe parenchymatous degeneration. Microscopically fatty degeneration severe. Glycogen + + +, diffuse.

Case 27.—Female, 55 years. One and a half years diabetic symptoms, on diet and insulin 22 units b.d. Insulin stopped for ? three days. Admitted in coma, with blood sugar 0.8%. Insulin 60 units at once, two hours later 100 units and after further two hours 100 units. Blood sugar one hour after last injection 0.9%. Patient died about half an hour after this.

P.M., 236/31, 14 hours after death. Body weight 80 lb. Liver (3 lb. 4 oz.) showed parenchymatous degeneration. Microscopically fatty degeneration moderate. Glycogen + + + + +, diffuse.

Case 28.—Male, 69 years. Five years diabetic, admitted in coma with blood sugar 0.7%. Insulin 60 units one and a half hours before admission, then 20 units hourly for four hours followed by 40 units hourly for four hours. No response. Blood sugar 1.0% half an hour before death.

P.M., 350/34, 27 hours after death. Body weight 129 lb. Liver (3 lb. 6 oz.) was parenchymatous and showed fatty degeneration. Microscopically fatty degeneration severe. Glycogen + + + +, diffuse with accentuation round portal systems.

Case 29.—Female, 25 years. No previous diabetic history, admitted in coma, blood sugar 0.5%. Insulin 100 units at once, followed by 400 units over next six hours. No effect, died seven hours after admission.

P.M., 219/35, nine hours after death. Body weight 82 lb. Liver (3 lb. 2 oz.) showed parenchymatous degeneration. Microscopically fatty degeneration moderate. Glycogen + + + +, periportal and midzonal, almost absent round central veins.

Case 30.—Female, 21 years. Known diabetic, admitted in coma when blood sugar 0.52%. Insulin 40 units two-hourly for six hours (120 units in all). No effect, died seven hours after admission.

P.M., 87/45, 24 hours after death. Body weight 100 lb. Liver (5 lb. 11 oz.) showed slight central congestion. Microscopically fatty degeneration severe. Glycogen + + +, diffuse. Liver fixed in Bouin's fluid.

Case 31.—Male, 21 years. Known diabetic, admitted in coma. Blood sugar 0.5%. Insulin 80 units intravenously. No effect, died one and a half hours after admission.

P.M., 226/46, seven hours after death. Body weight 116 lb. Liver (7 lb. 8 oz.) showed parenchymatous degeneration. Microscopically fatty degeneration severe. Glycogen + + + + +, diffuse with slight periportal accentuation.

Control Series

The following patients died of debilitating diseases. All necropsy findings reported in this series are macroscopic except for liver glycogen.

No. 1.—Female, 21 years. Heart failure, septicaemia, acute fibrinous on chronic rheumatic carditis, pregnancy.

P.M., 434/14, 18 hours after death. Liver anaemic and oedematous. No glycogen.

No. 2.—Male, 60 years. Oedema of glottis, pernicious anaemia.

P.M., 368/15, 24 hours after death. Liver showed slight enlargement, "creamy chocolate." No glycogen.

No. 3.—Male, 49 years. Double actinomycotic emphysema. Actinomycosis of wall of left chest.

P.M., 388/15, 20 hours after death. Liver showed parenchymatous degeneration. Glycogen ++.

No. 4.—Male, 59 years. Heart failure, subacute nephritis.

P.M., 111/26, 16 hours after death. Liver showed pinhead flecks of fibrosis in capsule, central congestion. No glycogen.

No. 5.—Female, 34 years. Serofibrinous pericarditis, slight cardiovascular hypertrophy, subacute nephritis.

P.M., 143/26, 18 hours after death. Liver showed oedema and central congestion. Glycogen +.

No. 6.—Female, 34 years. Oedema and collapse of lungs, ileus, pelvic peritonitis. Operation, caesarean section. Died six days following operation.

P.M. 174/34, 20 hours after death. Liver slightly enlarged and pale. No glycogen.

No. 7.—Female, 33 years, meningeal and cerebral haemorrhages, thrombocytopenic purpura.

P.M., 244/34, 16 hours after death. Liver showed oedema and diffuse congestion. Glycogen +.

No. 8.—Female, 22 years. Solid oedema of lungs, heart failure, Graves's disease and pregnancy (admitted in heart failure and died in three days).

P.M., 415/34, 18 hours after death. Liver showed parenchymatous degeneration. Glycogen ++.

No. 9.—Male, 41 years. Acute bronchitis, carcinoma of duodenum at ampulla of Vater. Old operation, cholecystgastrostomy.

P.M., 139/47, 30 hours after death. Liver showed parenchymatous degeneration. Glycogen +.

No. 10.—Male, 69 years. Fractured skull, purulent meningitis. Improved for six days, then died in 36 hours.

P.M., 149/47, eight hours after death. Liver showed slight fatty degeneration. No glycogen.

No. 11.—Male, 33 years. Cerebral herniation, spongioblastoma of right temporal lobe. Died nine hours after exploration.

P.M., 287/48, eight hours after death. Liver showed slight central congestion. Glycogen +.

No. 12.—Male, 59 years. Peritonitis, leaking caecostomy, carcinoma of pelvirectal junction.

P.M., 310/47, 15 hours after death. Liver showed accentuation of portal systems. No glycogen, but nuclear glycogen marked.

No. 13.—Female, 37 years. Multiple emboli, infarction of lung, mitral stenosis, and heart failure.

P.M., 55/48, 24 hours after death. Liver showed congestion. No glycogen.

The following are brief reports of patients dying quickly from acute medical conditions or as a result of accidents.

No. 14.—Male, 55 years. Acute heart failure, syphilitic aortitis with obstruction to coronaries. Brought in dead by police.

P.M., 489/14, 18 hours after death. Liver showed slight oedema with diffuse congestion. Glycogen ++.

No. 15.—Male, 62 years. Heart failure, cardiovascular hypertrophy, chronic nephritis. Admitted with heart failure and found to have cloud of albumin in urine. Reacted well to digitalis with improvement

in dyspnoea and loss of oedema. Died suddenly while walking in the ward.

P.M., 373/19, 36 hours after death. Liver showed slight portal fibrosis ; diffuse congestion. Glycogen + +.

No. 16.—Male, 47 years. Death under anaesthetic for removal of carcinoma of right tonsil, syphilitic aortitis (mild).

P.M., 589/19, 18 hours after death. Liver showed slight oedema, diffuse congestion. Glycogen + + +.

No. 17.—Female, 53 years. Cerebral haemorrhage, nephritis repens. Suddenly collapsed in the street, died soon after admission.

P.M., 269/31, 17 hours after death. Liver showed oedema. Glycogen + + +.

No. 18.—Male, 61 years. Heart failure. Organizing infarct of myocardium, thrombosis of atheromatous coronary arteries. Two and a half years angina of effort ; sudden severe attack of substernal pain ; died four hours later.

P.M., 172/34, seven hours after death. Liver showed oedema and slight focal congestion. Glycogen + +.

No. 19.—Male, 56 years. Pulmonary embolus, thrombosis in iliac veins. Organizing lobar pneumonia. Nine days following crisis, when convalescent, suddenly called for bedpan and died.

P.M., 434/34, 16 hours after death. Liver showed slight congestion, pale. Glycogen +.

No 20.—Male, 47 years. Brought in dead. Acute heart failure. Slight stenosis of coronaries by atheroma. Had pain in chest for one day, suddenly collapsed.

P.M., 155/47, six hours after death. Liver showed diffuse congestion. Glycogen + +.

No. 21.—Male, 45 years. Fracture of cervical vertebrae. Died in three hours following accident.

P.M., 158/47, 24 hours after death. Liver showed slight congestion. Glycogen + +.

No. 22.—Male, 66 years. Acute on chronic heart failure. Old cardiac infarction. One and a half years before cardiac infarction, good recovery. Sudden chest pain with acute distress, died four hours after admission.

P.M., 289/47, seven hours after death. Liver showed central congestion. Glycogen + +.

No. 23.—Male, ? 60 years. Brought in dead. Acute heart failure, severe stenosis of coronary arteries by atheroma.

P.M., 294/47, 22 hours after death. Liver showed severe central congestion. Glycogen + + +.

No. 24.—Male, 46 years. Cerebral haemorrhage, cardiovascular hypertrophy, hypertension. Sudden coma one and a half hours before admission, died in three hours.

P.M., 304/47, 12 hours after death. Liver normal. Glycogen + + +.

No. 25.—Male, 25 years. Fractured skull, posterior dislocation of right head of femur. Died 10 hours after the accident.

P.M., 68/48, five hours after death. Liver showed oedema. Glycogen + + +.

Both in the diabetic and control series all the livers in which glycogen could be demonstrated were found to be devoid of it after treatment with saliva.

J. clin. Path. (1952), **5**, 54.

PRIMARY AMYLOIDOSIS WITH RENAL AND MYOCARDIAL FAILURE

BY

A. WYNN WILLIAMS

From the Department of Morbid Anatomy, St. Mary's Hospital, London

(RECEIVED FOR PUBLICATION JUNE 29, 1951)

In secondary amyloidosis the kidney is one of the commonest sites in the body for the deposition of amyloid, and renal failure is a common sequel. In primary amyloidosis renal involvement is less common and renal failure exceptional. Only four cases of renal failure due to primary amyloidosis have been described in the literature. The individual patients of Christian (1932), Gerber (1934), and Lindsay (1948) respectively were males whose average age at the time of death was 57 years. The average duration of their illness was three years. The case of Platt and Davson (1950) was in a 60-year-old woman whose illness lasted 18 months.

It has been stated (Dahlin, 1950) that death from myocardial involvement occurs in roughly half the cases of systemic primary amyloidosis.

The description that follows is concerned with the case of a middle-aged woman who developed progressive renal and myocardial failure due to primary amyloidosis.

Case History

The patient was an unmarried post-office worker. At the time of her death she was 49 years of age. Her health had always been good except for diphtheria at the age of 6 years and quinsy at the age of 35 years. Her illness began two years before death with attacks of precordial pain brought on by exercise. Her doctor treated her for anaemia and the pain completely disappeared. Three months later, however, the pain returned and the patient also noticed swelling of the ankles. At this time (July, 1949) she was admitted to hospital.

Clinical examination revealed gross oedema of the legs and lower trunk. The blood pressure varied between 118/92 and 140/100 mm. Hg. The heart appeared normal, but electrocardiograms showed a slightly increased P–R interval and low voltage waves.

Laboratory Investigations.—Haemoglobin was 5.5 g. per 100 ml. (40% Haldane), red cells 2.4 million per c.mm., white cells 6,000 per c.mm. Total serum cholesterol was 510 mg. per 100 ml. The serum albumin varied between 0.8 and 3.5 g. per 100 ml. and the serum globulin between 1.4 and 1.8 g. per 100 ml. The fibrinogen estimations averaged 850 mg. per 100 ml. The gamma globulin was less than 100 mg. per 100 ml. on the average. The erythrocyte sedimentation rate was 67 mm. in the first hour (Wintrobe). Using defibrinated blood, the reading was 29 mm. The Wassermann and Kahn reactions were negative. The blood urea was 60 mg. per 100 ml. The urine was sterile but contained much albumin, an occasional red cell, and fairly numerous granular and hyaline casts. The urea clearance was about 50% of normal. The basal metabolic rate was −6%.

A diagnosis of Ellis type 2 nephritis was made and the patient was treated with rest, high protein/low salt diet, and injections of mersalyl. After three months in hospital the

oedema subsided. For a time the patient was able to undertake part-time work, but she suffered increasing dyspnoea on exertion as well as pain in the chest. The oedema of the ankles returned.

She was readmitted in April, 1950, with extreme shortness of breath and continuous vomiting. It was evident that there was now severe renal failure. Oedema extended up to the mid-thoracic region posteriorly and up to the inguinal ligaments anteriorly. The patient also had large pleural effusions and papilloedema. The blood pressure was 130/75 mm. Hg. The pulse was 60 and regular. There was a systolic murmur at the left border of the sternum and a diastolic murmur at the pulmonary area. Electrocardiograms showed evidence of considerable myocardial damage with almost complete A–V block, and a marked lowering of the voltage in all leads. There was some enlargement of the thyroid. The haemoglobin was 6.9 g. per 100 ml. blood (50% Haldane), the blood urea 280 mg. per 100 ml., the total serum cholesterol 710 mg. per 100 ml., the serum albumin 2.1 g. per 100 ml., and the serum globulin 3.4 g. per 100 ml. The erythrocyte sedimentation rate was 146 mm. in the first hour (Wintrobe, uncorrected for fibrinogen).

On May 4, 1950, the patient was transfused with one pint of group O blood. On May 20, 1950, 2,400 ml. pale, serous fluid was aspirated from the pleural sacs. The fluid quickly re-accumulated. The high protein/low salt diet was continued, and Shohl's solution was administered to combat acidosis. In the last month before death the urine contained traces of glucose. The patient died on July 25, 1950. The necropsy was performed on the following day.

Post-mortem Findings

The body was that of a middle-aged female 1.6 m. in length and 80 kg. in weight. There was marked generalized oedema. The head and neck were cyanosed. Petechial haemorrhages were numerous over the front of the neck and a few were present on the face. The tongue appeared normal.

The heart weighed 440 g. Fibrinous pericarditis was present. All chambers were dilated. Both ventricles were thickened. The myocardium was of normal consistency but paler than usual. The left ventricle showed some fatty degeneration. At and below the line of closure of the mitral and aortic valve cusps were numerous rounded, grey-pink, semi-translucent deposits (Fig. 1). They were smooth or slightly roughened and measured 1–2 mm. in diameter. Some had apparently coalesced into masses measuring 4–6 mm. in diameter. They did not appear to be covered with fibrin and they could not be dislodged without tearing the cusps. Apart from these lesions, the mitral and aortic cusps showed some slight fibrous thickening. The coronary arteries were slightly thickened. There was severe atheroma in the abdominal aorta.

Massive pleural effusions had produced marked collapse of the lungs, which were very oedematous. Small, fibrous adhesions were present at the apices of both upper lobes.

About a litre of serous fluid occupied the peritoneal cavity. The liver was enlarged (1,772 g.) and showed nutmeg congestion. The intestines were congested and showed also small, scattered subperitoneal haemorrhages.

The spleen was enlarged (250 g.) and congested. The lymph nodes were normal except for calcification in two small ileo-caecal nodes.

With the exception of a unilocular cyst, containing watery fluid, in the upper pole of the right kidney, each kidney had the same features: reduction in volume

and weight (right kidney 96 g., left kidney 104 g.); the capsule stripped easily, revealing a finely granular surface; the cortex was pale and reduced in thickness (average thickness 3 mm.) but still well demarcated from the medulla; the interlobar and interlobular arteries were thickened, but there was no abnormality of pelvis, ureters, or bladder.

A few small fibromyomata

FIG. 1*a*

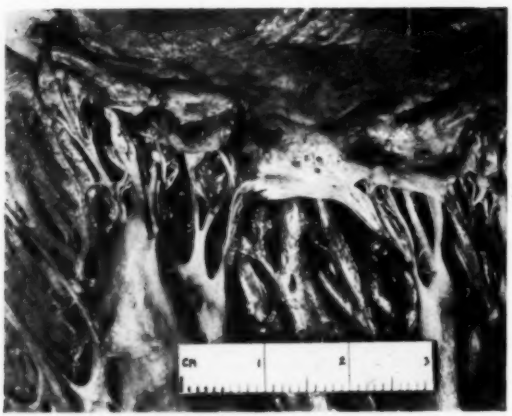


FIG. 1*b*

FIG. 1*a*.—Amyloid deposits in aortic cusps.

FIG. 1*b*.—Amyloid deposits in mitral cusps.

were present in the uterus. The whole of the vaginal aspect of the external os was reddened by an "erosion." Both ovaries had numerous superficial haemorrhages. The breasts were normal. The adrenals contained plentiful cortical lipoid. The left lobe of the thyroid was slightly enlarged. Both lobes were firmer than normal. On section the thyroid was greyish-pink and less glistening than normal. The brain (1,250 g.) was slightly oedematous. No abnormality was discovered in the joints or skeletal muscles.

Microscopic Findings

The subendocardial deposits consisted of masses of amyloid (Fig. 2*a*). The staining reactions are described later. The fibrinous pericarditis was confirmed. In the myocardium there was a variable degree of amyloid (Figs. 2*b* and 2*c*). A frequent finding was an amyloid investment of muscle fibres, which were often atrophied. Some fibres had been wholly replaced by amyloid, others had undergone hypertrophy. The walls of the larger myocardial blood vessels were thickened by amyloid. No amyloid was present in the largest arteries in the body, e.g. the carotids.

FIG. 2*a*

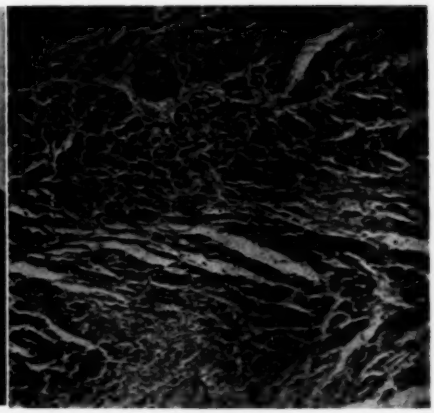


FIG. 2*b*

FIG. 2*a*.—Subendocardial amyloid deposits. The clefts are artefacts. Congo red × 40.

FIG. 2*b*.—Amyloid deposits in the myocardium. Note the involvement of the arteriole. Haematoxylin and eosin × 70.

FIG. 2*c*.—Amyloid deposits in the myocardium. There is replacement of muscle fibres by amyloid. Congo red × 200.

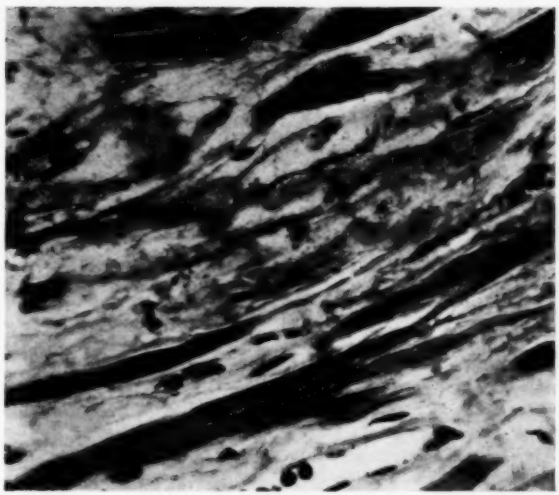


FIG. 2*c*

The lungs were markedly oedematous. Pulmonary arterioles, venules, and capillaries frequently showed amyloid thickening. Small patches of amyloid were also scattered irregularly in the alveolar walls.

The arteries and veins in the portal tracts of the liver were markedly thickened by amyloid (Fig. 4). No amyloid was present in the lobules themselves. There was pronounced centrilobular congestion. In the spleen the sinuses were dilated from chronic venous congestion. Some of the Malpighian corpuscles were largely replaced by amyloid (Fig. 5). There was also slight amyloid thickening of the blood vessels, mostly arterioles, and patchy amyloid deposits were present in the trabeculae.

In the kidneys there was amyloid infiltration, more or less severe, of all glomeruli (Fig. 3). Those that were most affected were much larger than normal. In

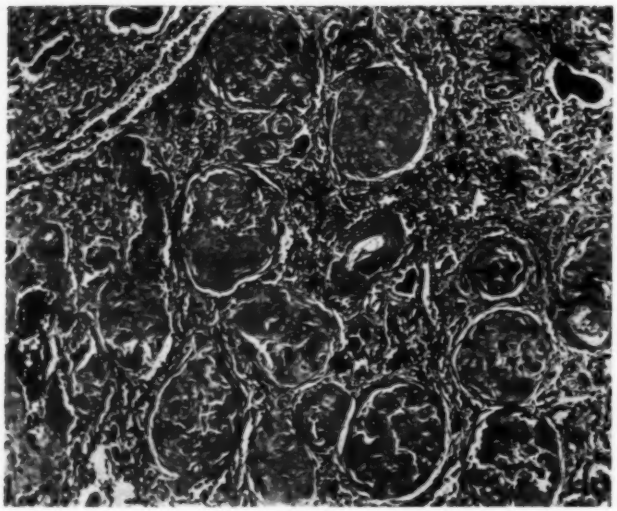


FIG. 3

FIG. 3.—Considerable amyloid deposit in the glomeruli of the kidney, also in the blood vessels. Congo red × 70.

FIG. 4.—There is considerable amyloid thickening of the blood vessels in the portal tracts of the liver. No amyloid is present in the lobules. Haematoxylin and eosin × 70.

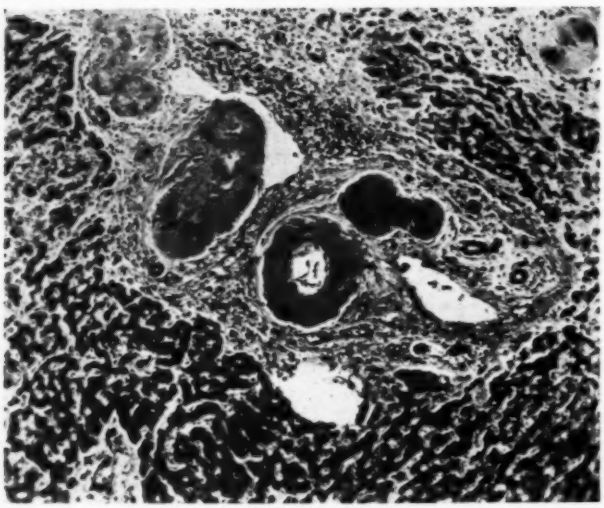


FIG. 4

FIG. 5.—Amyloid deposit in Malpighian corpuscle of the spleen. Haematoxylin and eosin × 70.

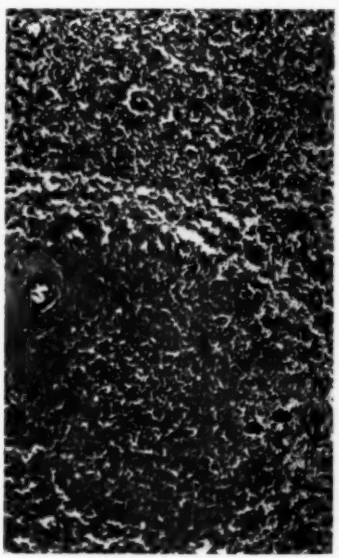


FIG. 5

addition, there was marked amyloid thickening of arterioles, some arteries and, to a lesser degree, of some venules and capillaries. As in the liver, all the coats of the larger vessels were affected. Many tubules had disappeared entirely. Those that remained were often dilated and frequently were lined by atrophic epithelium. Small amounts of fat were present in the tubules, especially the convoluted parts, and in the glomeruli. Hyaline casts were frequent in the tubules, and moderate numbers of polymorphonuclear leucocytes were present in some tubules, though they were not seen in the interstitial tissues of the kidney. A small amount of fibrosis had occurred in many glomeruli and a larger amount in the interstitial tissue, but there was no special fibrous thickening around Bowman's capsule.

Arteries in the pericapsular connective tissue of the adrenals, as well as in the adrenal medulla, were thickened by amyloid. The ovaries showed marked amyloid thickening of arteries, also of some of the smaller blood vessels. They also showed small recent haemorrhages.

In the stomach and intestines there was amyloid thickening of submucosal arteries.

Sections of the thyroid showed scanty acini separated by broad bands of interstitial tissue containing abundant amyloid (Fig. 6). The acinar lining was often atrophic and colloid was very scanty. Some amyloid thickening of vessels, mainly veins, was present.

Most lymph nodes were normal. In a few of them there was some amyloid thickening of the arteries.

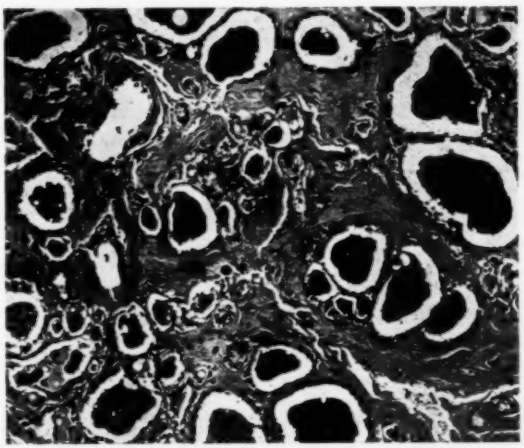


FIG. 6.—Considerable amyloid deposit in the connective tissue of the thyroid. Haematoxylin and eosin × 70.

There was slight patchy amyloidosis of the fibrous connective tissue in the tongue. The arteries of many skeletal muscles were slightly thickened by amyloid.

In the salivary glands there was amyloid thickening of the arteries and of the basement membranes of ducts and some acini.

Sections of the brain revealed no abnormality.

Positive staining reactions for amyloid were given by iodine, iodine green, P.A.S. (McManus, Hotchkiss, modified by Pearse), silver (modified Hortega, after King), and Congo red. The amyloid material also stained metachromatically with methyl violet and crystal violet.

The preparations stained by Congo red deserve special mention because it was observed that the combination of amyloid and Congo red was doubly refractile, as described by Ladewig in 1945. Only patches of the amyloid material were doubly

refractile, however, and there was no correlation between the density of Congo red staining and the emergence of double refraction.

Discussion

Wild (1886) reported the first case of primary amyloidosis, but it received little attention until the classical papers of Königstein (1925) and Lubarsch (1929). It differs from secondary amyloidosis by the absence of demonstrable pre-existing disease, by a special tendency toward the involvement of the heart, lungs, muscles, skin, and other structures not often or seriously affected in secondary amyloidosis, and by its staining reactions, which are sometimes atypical. It is of interest that it occurs in animals, particularly in horses and dogs.

Approximately 70 cases of primary systemic amyloidosis have appeared in the literature. The vast majority were not diagnosed until necropsy. In a recent review of 55 cases Dahlin (1950) reported death from cardiac involvement in at least 14 examples. Lesions of the skin and skeletal muscles were present in more than a fourth of the cases. Several patients had purpura. The liver, spleen, kidneys, and adrenals contained amyloid in more than a third. In three patients, all males, severe renal failure occurred. Christian's patient (case reported in 1932) was a man of 53 who died of bronchopneumonia after an illness lasting nearly three years. He had generalized oedema with marked albuminuria and a reduced total plasma protein with reversal of the normal albumin-globulin ratio. The blood pressure and blood urea were normal. The basal metabolic rate was subnormal. Occasional hyaline and granular casts were present in the urine. At necropsy amyloid was present in the kidneys, spleen, liver, and adrenals. The right kidney weighed 295 g. and the left 300 g. Both kidneys were oedematous. The capsule stripped easily. There was marked deposition of amyloid in the glomeruli. Marked tubular degeneration, with fatty change, was present. The blood vessels are reported as showing " hyaline " change. There was patchy interstitial fibrosis, and occasional casts were present in the tubules.

The case reported by Lindsay (1948) resembles Christian's closely except for the addition of amyloidosis of the heart. The patient was a man of 66 who was ill for two years. He had generalized oedema and severe albuminuria. Occasional hyaline and granular casts were seen in the urine, as well as a few white cells. The plasma albumin was markedly reduced. The blood cholesterol was raised. The blood pressure and basal metabolic rate were normal. Electrocardiograms showed low voltage curves. The patient died of pulmonary oedema and pulmonary infarction. At necropsy extensive amyloid deposits were present in the heart, in the sub-epicardial fat, throughout the myocardium, and beneath the endocardium of the atria and tricuspid valve. Deposits were also found in the pulmonary vessels and in many other visceral blood vessels, e.g. in the liver, gall-bladder, gastro-intestinal tract, kidneys, pancreas, ovaries, pituitary, thyroid. Arteries, arterioles, veins, venules, and capillaries were all affected. Amyloid deposits were especially noted under the endothelium of arteries. A slight deposition of amyloid was noted in the dermis.

The right kidney weighed 240 g. and the left kidney 250 g. The outer surface was slightly granular and pale yellow. The capsule was slightly adherent. The

cortico-medullary junction was ill-defined. There was much deposition of amyloid in the glomeruli, but only a few of the latter were completely replaced by amyloid. Subendothelial amyloid was seen in afferent arterioles and in some smaller vessels in the medulla and pelvis. There was severe tubular damage and lipoid droplets were frequent in the degenerate epithelium. Small deposits of amyloid were present in the interstitium of the cortex as well as collections of lymphocytes.

The report of Gerber (1934) concerned a 51-year-old Russian Jew who was ill for three years. The patient had complained of pruritus of the thighs and vague pains and weakness in the back and thighs. He was found to have marked albuminuria and an enlarged liver. Doubly refractile crystals and a few white and red cells were found in the urine, and slight glycosuria was also reported. The blood pressure and plasma proteins were normal; the blood cholesterol was raised; the basal metabolic rate was subnormal. The patient died in uraemia with pulmonary oedema. At necropsy there was extensive amyloid deposit in the vertebrae, with partial collapse of the bodies of the ninth thoracic and first lumbar vertebral bodies, and in the iliac bones, femur, and ribs. The liver showed diffuse amyloid deposits, with involvement of blood vessels. Similar deposits were found in the pancreas, spleen, kidneys, suprarenals, thyroid, and stomach mucosa. The right kidney weighed 120 g., the left 110 g. Both kidneys showed the same features: finely-granular outer surface, waxy cut surface, normal-looking cortico-medullary junction; the glomeruli were largely replaced by amyloid, and amyloid was present in the media of the small and medium-sized vessels as well as in the basement membranes of the tubules; the larger vessels were free of amyloid; there was considerable tubular atrophy and degeneration with dilatation of many surviving tubules and fibrosis of the interstitial tissue.

Platt and Davson in their study of renal disease (1950) refer briefly to a case of primary amyloidosis with renal failure in a woman of 60. She had been ill for 18 months with weakness, tiredness, loss of weight and appetite, shortness of breath on exertion, and swelling of the legs. Examination showed evidence of myxoedema as well as generalized oedema, enlargement of the liver and spleen, hypertension (150/100), albuminuria, and a mild hypochromic, normocytic anaemia. The serum albumin was 3.1 g. per 100 ml. and the serum globulin 2.8 g. per 100 ml. The blood urea was 180 mg. per 100 ml., rising to 232 mg. per 100 ml. before she died "apparently in uraemia." At necropsy the ventricles of the heart were seen to be hypertrophied, the spleen was enlarged (750 g.), and the kidneys (150 g. and 85 g.) were finely granular and showed loss of cortical pattern and yellow lipoid deposition in the boundary zone. Histologically, amyloid was present in the liver, spleen, lung, thyroid, adrenals, kidneys, pancreas, myocardium, and aorta, but no details are given. This case differs from those already mentioned in that the kidneys were so different in weight. In the absence of histological details in this case the inequality of the kidneys raises doubt as to the "primary" nature of the amyloidosis, since it seems possible that one kidney might have been the seat of old-standing pyelonephritis.

The case reported in this paper affected a woman aged 49. Clinically, her condition resembled that of Lindsay's patient and Platt and Davson's patient. On histopathological grounds, the widespread amyloidosis of the heart and blood vessels was an additional point of similarity to Lindsay's patient; but, whereas the kidneys

in Lindsay's and Christian's cases were enlarged, the kidneys of the case here reported resembled those of Gerber's patient in being reduced in size.

Christian made no comment on the distribution of amyloid in the walls of blood vessels. Platt and Davson, apart from referring to the presence of amyloid in the aorta of their patient, gave no details concerning the distribution of the amyloid within the wall of that vessel. Lindsay referred to deposits of amyloid in the subendothelial part of arteries. Gerber mentioned special involvement of the media of the renal arteries. In my own case most arteries affected by amyloid showed deposits throughout their walls, but in some arteries the amyloid was confined almost entirely to the media.

Summary

A case of primary systemic amyloidosis in a woman of 49 is described. The illness lasted two years and was accompanied by progressive renal and myocardial failure. The amyloid was mainly of cardiovascular distribution.

Reference is made to the four other cases of primary systemic amyloidosis with renal failure reported in the medical literature.

I am grateful to Dr. Geoffrey Bourne for permission to mention clinical details, to Professor Dorothy S. Russell and Professor W. D. Newcomb for advice, and to Mr. Alec Beasley and the photographic unit, St. Mary's Hospital, for technical help.

References

Christian, H. A. (1932). *Med. Clin. N. Amer.*, **15**, 805.
Dahlin, D. C. (1950). Ibid., **34**, 1107.
Gerber, I. E. (1934). *Arch. Path., Chicago*, **17**, 620.
Königstein, H. (1925). *Arch. Derm. Syph., Wien*, **148**, 330.
Ladewig, P. (1945). *Nature, Lond.*, **156**, 81.
Lindsay, S. (1948). *Amer. J. Med.*, **4**, 765.
Lubarsch, O. (1929). *Virchows Arch.*, **271**, 867.
Platt, R., and Davson, J. (1950). *Quart. J. Med.*, n.s., **19**, 33.
Wild, C. (1886). *Beitr. path. Anat.*, **1**, 175.

J. clin. Path. (1952), **5**, 63.

BONE-MARROW EMBOLISM

BY

R. B. H. TIERNEY

From the Pathology Department, St. Bartholomew's Hospital, Rochester

(RECEIVED FOR PUBLICATION OCTOBER 10, 1951)

The finding of emboli of haemopoietic tissue in the lungs is not uncommon in experimental animals, but is extremely rare in man. Most of the reported cases have been associated with fractures, and in the remainder convulsions preceded death. Bone-marrow embolism was first demonstrated by Lubarsch (1898) and Lengemann (1897) in rabbits following the injection of liver, kidney, or placental cells into the jugular vein. Lubarsch was also the first to observe bone-marrow emboli in human beings, in four women who had died from eclampsia. In all these cases haemopoietic tissue was transported without any destruction of bone, a fact which Lubarsch emphasized in opposition to Maximow (1898), who, in his experiments on rabbits, saw such emboli only after extensive fragmentation of bone. Lubarsch laid great stress on local hyperaemia and softening of the bone-marrow as predisposing factors, though he admitted that in human beings some shock to the bone-marrow might be needed as well. Lengemann (1901), while agreeing that some alteration of the bone-marrow was a predisposing factor, attached greater importance to concussion of bone in both man and experimental animals, and considered that the violent movements of the latter during manipulation played some part. The reports of these early German workers, and some experimental work on the ultimate fate of these emboli, were reviewed by Ceelen (1931), who added that no sequels of any importance followed bone-marrow embolism, for even if small or medium sized arteries were blocked a collateral circulation developed.

Since that time only a few reports have appeared in the literature. Sotti (1910), reporting a necropsy on a woman of 78 who died of senile dementia in a mental hospital, described the finding of a bone-marrow embolus in the lung ; although he gave no further clinical history it is probably reasonable to assume that convulsions preceded death. Karlén (1942) found fat and bone-marrow emboli in the lungs of a patient who died in convulsions following intradural " perabrodil " myelography. The same observations were made by Schenken and Coleman (1943) in a woman of 86 who died after the operative reduction of a fractured neck of femur. Lindsay and Moon (1946) reported three cases following multiple fractures. Warren (1946) observed marrow particles in the pulmonary arteries in three out of 100 cases of fat embolism. Recently Rappaport, Raum, and Horrell (1951), in a study based upon cases in the files of the Armed Forces Institute of Pathology, Washington, reported 27 new cases ; of these two were personally observed by the authors, another five were found in the files with the diagnosis of bone-marrow embolism, and the remainder were brought to light by a systematic survey of cases in which death followed trauma with multiple fractures, accidental electrocution, and generalized

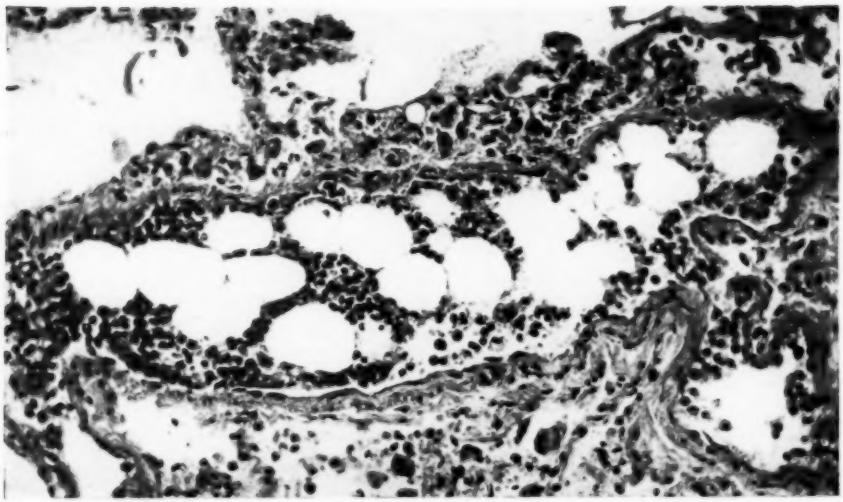


FIG. 1.—An embolus of fat cells and haemopoietic tissue filling a branch of the pulmonary artery (haematoxylin and eosin, × 190).

convulsions, and in which specimens and sections were available for study. Of the 27 cases, 18 were instances of post-traumatic death, two of accidental electrocution, and seven of death following severe generalized convulsions. These cases bring the total number recorded in the literature to 40.

The following case of fat and bone-marrow embolism following crush injuries of the ankles has recently been encountered.

Case Report

A healthy man, aged 44 years, was engaged in operating an overhead crane and grab, moving coal from a large heap and depositing it over a conveyor belt. While he was making some adjustments the brake slipped, the grab descended to the ground, and he was trapped by his legs between two steel rollers which carry the cables operating the grab. These rollers are only 2¼ in. apart, and the securing bolts had to be cut by oxy-acetylene flame to free him. He was admitted to hospital one hour after the accident. Respirations were then found to be absent, but the radial pulse was perceptible for about ten minutes.

Necropsy.—This was performed for the coroner by the casualty officer. He found severe crush injuries of both ankles, involving both soft tissues and bones. There was also widespread laceration of the soft tissues in the lumbar region. No other bony injuries were found. Nothing abnormal was found in the brain, heart, liver, kidneys, spleen, or alimentary tract. There were some old pleural adhesions at the right base, and a small piece of the right lung was submitted to the laboratory for histological examination. There is unfortunately no record of the naked-eye appearance of the lungs. Death was attributed to primary shock.

Histology.—Paraffin sections of the lung stained with haematoxylin and eosin showed intense capillary congestion with widespread intra-alveolar haemorrhages, and alter-

nating zones of oedema and emphysema. Apart from many dust-laden phagocytes there was no cellular reaction. The most striking feature, however, was the presence of fragments of haemopoietic tissue in branches of the pulmonary artery; some of these were sufficiently large to make their nature obvious and included several fat cells (Fig. 1), but careful search revealed many smaller fragments, and individual marrow cells were scattered widely throughout the pulmonary arterioles and capillaries. Frozen sections stained with Sudan IV confirmed a gross degree of fat embolism.

Discussion

It is evident that bone-marrow embolism may occur in two distinct types of injury. In the first place convulsions may impart such a shock to the skeleton as to dislodge fragments of haemopoietic tissue. Secondly it may follow fracture of bone, as in the present case and most of those reported, the marrow fragments being forced into the veins by the increased pressure resulting from local haemorrhage and oedema. Most of the traumatic cases have been road, railway, or aeroplane accidents in which fractures were multiple, and in these circumstances the occurrence of bone-marrow embolism is not really surprising. In trauma of lesser degree, however, the very nature of the injury may be a predisposing factor. Schenken and Coleman, for example, whose patient died four days after the operative reduction of a fractured neck of femur, postulated that fragments of marrow may have been forced into the veins mechanically by the wooden bone screws which were used; and in the present case it is tempting to suppose that a similar mechanical action resulted from the squeezing of the ankles between the steel rollers.

It is noteworthy that bone-marrow embolism is by no means invariably accompanied by fat embolism, that is to say embolism by extracellular fat. Rappaport *et al.* found no evidence of fat embolism in nine out of 21 of their cases in which suitable material was available for study, nor did they observe any direct relationship between the severity of fat and bone-marrow embolism. These workers also present some interesting figures on the incidence of bone-marrow embolism; they found 13 instances (6%) in a survey of 203 consecutive cases with multiple fractures and none in a control series of 240 necropsies. Thus it seems likely that a greater awareness of this condition among pathologists will lead to its detection more frequently than hitherto. Their main thesis, however, concerns the mechanism of bone-marrow embolism in deaths following generalized convulsions. They consider that detachment of marrow fragments by concussion alone is unlikely, and suggest that in those cases of their series in which convulsions preceded death bone-marrow embolism was due to undetected vertebral fractures, a suggestion which Karlén had also made in his case. This is an intriguing hypothesis, for indeed a crush fracture of a vertebra might well predispose to bone-marrow embolism by its very nature. Anatomical proof is not easy to obtain, even if looked for, at necropsy. This problem can be solved only in a post-mortem room where radiographic facilities are available, and it is proposed to make it the basis of a future study.

Summary

A case of fat and bone-marrow embolism following crush injuries of the ankles is described.

The literature on bone-marrow embolism is reviewed.

The pathogenesis of this condition is discussed.

I am indebted to Mr. T. H. Church, H.M. Coroner for Gravesend, for allowing me access to his records.

References

Ceelen, W. (1931). In Henke, F., and Lubarsch, O., *Handbuch der Speziellen Pathologischen Anatomie und Histologie*, vol. 3 (iii), p. 105. Berlin.
Karlèn, A. (1942). *Acta chir. scand.*, **87**, 497.
Lengemann, P. (1897). Ueber die Schicksale verlagerter und embolisierter Gewebsteile im tierischen Körper. (Inaugural Dissertation, Rostock.) Wiesbaden. Reprinted in Lubarsch, O., *Zur Lehre von den Geschwülsten und Infectionskrankheiten*, 1899, pp. 3–73. Wiesbaden.
—— (1901). *Beitr. path. Anat.*, **29**, 1.
Lindsay, S., and Moon, H. D. (1946). *J. Bone Jt Surg.*, **28**, 377.
Lubarsch, O. (1898). *Virchows Arch.*, **151**, 546.
Maximow, A. (1898). Ibid., **151**, 297.
Rappaport, H., Raum, M., and Horrell, J. B. (1951). *Amer. J. Path.*, **27**, 407.
Schenken, J. R., and Coleman, F. C. (1943). *Amer. J. Surg.*, **61**, 126.
Sotti, G. (1910). *Arch. Sci. med.*, **34**, 227.
Warren, S. (1946). *Amer. J. Path.*, **22**, 69.

J. clin. Path. (1952), **5**, 67.

CULTURE OF HUMAN BONE MARROW *IN VITRO*
THE REVERSIBILITY BETWEEN NORMOBLASTIC AND MEGALOBLASTIC SERIES OF CELLS

BY

L. G. LAJTHA

From the Department of Clinical Pathology, Radcliffe Infirmary, Oxford

(RECEIVED FOR PUBLICATION JULY 20, 1951)

Earlier publications have dealt with findings in human bone marrow culture (Rusznyák, Löwinger, and Lajtha, 1948 ; Cairns and Lajtha, 1948 ; Lajtha, 1950 ; Callender and Lajtha, 1951a and b). This communication is concerned with the description of the technique, and with the interrelationship between normoblastic and megaloblastic series of cells.

The Marrow Culture Technique

The classical methods of tissue culture *in vitro* are not necessarily ideal for investigations on bone marrow. Most of the emigrating cells from pieces of tissue cultured on a solid supporting framework develop the " fibroblast form," and indeed it is a limitation of the classical tissue culture technique that tissues which cannot develop the " fibroblast form " of cells cannot or are very difficult to culture *in vitro*.

More physiological conditions are ensured by culturing bone marrow in certain parts of the body (for example, in the anterior chamber of the eye or ear), but there are serious objections to these techniques also. First, under such circumstances cells can emigrate and immigrate from the culture " chamber," and changes in the distribution of the cells are just as difficult to evaluate as changes in marrow obtained at different intervals of time from the bones. Secondly, although direct observation of the cells by means of a phase contrast microscope is possible in these conditions, the value of microscopy (even that of phase contrast microscopy) is very much limited in differentiating between various types of living cells. For the purpose of bone marrow culture it is obviously essential not to disturb the normal differentiation of the cells, and to be able to observe the changes due to differentiation. It was, therefore, necessary to develop a special technique which would fulfil these requirements.

The principle of culture of cell suspensions was adopted. This has been found to allow similar processes of cell differentiation as occur in bone marrow *in vivo*, and allowed accurate total and differential counts on the cells. The technique is a modification of Osgood's procedure (Osgood and Brownlee, 1937).

Material.— (1) McCartney bottles (30 ml.) sterilized by autoclave with a hole (approximately 5 mm. diameter) punched in the metal caps. Since rubber is toxic to

the cultures, the rubber washers are dipped in collodion and dried overnight in an incubator before inserting them into the caps. The caps are then screwed on to the bottles and the bottles sterilized.

(2) Five 30-ml. all-glass syringes; ten 10-ml. all-glass syringes; ten 1-ml. all-glass syringes; 24 needles (No. 18 s.w.g.), 1½ in. long; 24 needles (No. 18 s.w.g.), 3 in. long. These are sterilized by dry heat and stored in glass tubes stoppered with cotton wool.

(3) Heparin-Ringer solution (1 : 20,000 heparin).

(4) Gey solution (Gey and Gey, 1936), made up as follows:

NaCl	8.00 (g./l.)
KCl	0.37
$NaHCO_3$	0.23
$CaCl_2$ anhydr.	0.17
$MgCl_2 6H_2O$	0.21
$Na_2HPO_4 2H_2O$	0.15
KH_2PO_4	0.03
$MgSO_4 7H_2O$	0.07
Dextrose	1.00

These solutions are Seitz-filtered and kept sterile in McCartney bottles in the refrigerator.

(5) Human serum obtained under sterile conditions is stored in McCartney bottles in the refrigerator. If possible serum older than 28 days should not be used.

Procedure.— Two ml. of sternal marrow are injected into 18–20 ml. of heparin-Ringer solution heated to 30–36° C. by piercing the rubber disc through the perforation in the cap. Within one hour the bottles are centrifuged for 10 minutes at 1,500 r.p.m. The supernatant fluid is sucked off with a syringe. The suction is facilitated by employing an air vent. Five to 10 ml. of Gey's solution at 30–36° C. are added to the cell suspension and the contents mixed thoroughly by pumping the fluid in and out with a syringe for at least three minutes. Less thorough mixing results in the cells clumping. One drop of the marrow suspension is sucked off and a preliminary count of the nucleated cells is made, using ordinary leucocyte-diluting fluid. The count should fall between 15,000 and 50,000 cells per c.mm. If it exceeds this range, adjustment is made by adding more Gey's fluid. To prepare the final culture medium it is now calculated how much serum and Gey's solution must be added to the suspension to get a mixture containing the desired concentration of serum (this can vary from 20 to 95%, depending on the design of the experiment) and approximately 2,000–4,000 nucleated cells per c.mm. If, for example, the suspension contains 30,000 nucleated cells per c.mm., and an 80% serum concentration in the medium has been decided upon, 1 in 10 dilution would be suitable. Hence 16 ml. of serum are mixed with 2 ml. of Gey solution in a syringe and 2 ml. of the suspension are sucked into the mixture.

If it is desired to investigate the influence of special substances, these should be dissolved or suspended in physiological saline in such concentration that not more than 0.10 ml. of the preparation has to be added to each 3 ml. of medium, i.e. 0.03 ml./ml. medium.

After careful mixing of the final culture in the syringe by shaking for two or three minutes a volume of 3 ml. is injected into each of a number of McCartney bottles. Sufficient fluid must be left in the syringe to allow an accurate total cell count to be made. This represents the initial or "start" count. The initial differential count is done on the slides prepared during the original sternal puncture. It was found that this

count does not differ significantly from differential counts made on smears from the cultures at 0 hours. It is considered necessary to count at least 500 nucleated red cells or, better still, 1,000.

The cultures are kept in an incubator at 37° C. and at desired intervals a bottle is taken out, the contents mixed thoroughly for three or four minutes, and an accurate total count of the nucleated cells made as before. The bottle is then centrifuged at 1,500 r.p.m. for 10 minutes and the supernatant fluid discarded, one or two drops being left to ensure a homogeneous mixture of the cells. Slides are now prepared and differential counts done as before. It may occasionally be advisable to open two bottles at one time as a control of the uniform behaviour of the cultures.

The marrow from a sternal puncture is best obtained by sudden forceful suction. A more cellular marrow less diluted with peripheral blood results from this method.

In experiments involving the culture of megaloblastic marrow in pernicious anaemia serum the serum should be taken from a different subject, since the patient's own serum may ripen megaloblasts to a greater extent than serum from another patient. This has been observed previously by Osgood and Brownlee (1937).

The method can naturally be adapted to the use of other media such as normal, dried, and reconstituted plasma. It has been observed that in normal plasma maturation and degeneration of the cells proceeds faster. Dried and reconstituted plasma lacks factors necessary for normal maturation, and in old cultures this plasma was sometimes found to be toxic. When serum is used the bone marrow cultures may be kept alive for three to five days and leukaemic cells for 10 to 14 days.

Changing the medium, which might theoretically prolong the life of the cultures, cannot be carried out for the following reasons. (1) It is impossible to replace exactly the amount of old medium drawn off. The statistical significance in the total counts is therefore impaired. (2) The new medium added will always be of different composition from the old fluid drawn off. This sudden change of medium is noxious to the cells. (3) Finally, it is doubtful whether more useful information can be obtained by keeping the cells alive for longer periods than are already assured by the simple method described above.

If possible it is advisable to employ serum of the same blood group as that of the patient from whom the culture is taken. Although the white cells are not affected by incompatible serum, homogeneous suspensions are difficult to obtain and the differential counts suffer accordingly.

The value of the method depends largely on the accuracy of the total counts. As many as possible of the components of error usually associated with the white cell count have therefore been eliminated by using the same pipettes and counting chambers throughout. The error due to filling the pipettes can be reduced by using two to four pipettes for each count. In these circumstances, if all counts are made by one observer, the error is found to approach that of the Poisson distribution. Thus for counts of 500 cells the coefficient of variation is about 5% and for 1,000 cells about 3%.

Silicone Coating.— It is known that white cells can "stick" to glass surfaces. Since large numbers of cells adhering to the surface of the culture bottles would modify the culture results, this problem needed investigation.

A grease-free coverslip was laid on the bottom of several culture bottles, and was removed, washed in running tap water, and stained at the same time as the smears and total counts were made. A large number of cells were found to be adhering firmly to the coverslip; the cells involved were always predominantly myeloid and monocytoid. No adhesion of nucleated red cells was ever seen. The density of the deposit was about 300,000 per sq. cm.

Parallel cultures were set up with ordinary and silicone-coated culture bottles. The results were as follows:

TOTAL NUCLEATED CELL COUNTS

Pipette No.	"Glass" 1	2	3	4	"Silicone" 5	6	7	8
Initial count	47	35	40	42	39	38	30	59
	37	43	39	34	42	40	29	52
	50	49	41	43	20	44	41	40
	40	39	30	45	42	44	41	40
			W.B.C.:		1,011			
6-hr. culture	35	47	35	45	44	51	47	60
	45	30	45	28	51	48	46	43
	40	42	32	47	50	37	31	47
	30	27	44	33	46	40	32	44
	W.B.C.: 945				W.B.C.: 1,120			

DIFFERENTIAL COUNTS ON 200 CELLS EACH

	Initial Count	6-Hour Culture in Glass	in Silicone
Myeloblasts	5	5	5
Promyelocytes	27	34	21
Myelocytes	144	233	100
Band cells	316	194	311
Polymorphs	277	228	353

These results indicated that, although the differential distribution of nucleated red cells is not altered by cells adhering to the surface of the culture bottles, the white cell distribution might be influenced by it. The cultures where total nucleated cell counts were performed were, therefore, cultured in silicone-coated bottles.

It should be noted that all the silicone must be hydrolysed to the glass surface, free silicone being very toxic for the cells.

Nomenclature.—The following nomenclature is used.

Pronormoblasts.—The first recognizable nucleated red cells. Large, strongly basophilic cytoplasm, fine nuclear structure, numerous nucleoli.

Basophil Normoblasts.—Smaller in size than pronormoblasts, nuclear structure coarser, fewer nucleoli.

Polychromatic Normoblasts.—Various degree of haemoglobination in the cytoplasm. Nucleus gradually changing from "finely lumpy" to pyknotic. No nucleoli.

Orthochromatic Normoblasts.—Usually pyknotic nuclei. No traces of basophilia in the cytoplasm; it has the colour of the ripe erythrocyte (and not the polychromatic erythrocyte!).

Megaloblasts.—The cells which are characteristic for pernicious anaemia in relapse and are never found in normal marrows or in marrows other than liver factor or folic acid deficiency (Ehrlich and Lazarus, 1898; Naegeli, 1931; Jones, 1943, 1947, 1948; Dacie and White, 1949).

Culture of Normoblastic Marrows *in vitro*

Preliminary experiments showed that with the marrow culture technique human bone marrows could be kept alive for several days. Before proceeding to the culture of megaloblastic bone marrows *in vitro* it was essential to establish how far the behaviour of human bone marrow in culture approaches that observed *in vivo*. For this purpose, studies were made on a series of 23 normal marrows from normal subjects cultured in normal serum.

The results are represented in Tables I and II and Figs. 1, 2, and 3.

The percentage of the early red cell precursors (pronormoblasts and basophilic normoblasts) which initially represent one-fourth to one-fifth of the nucleated red

TABLE I

CULTURES OF NORMOBLASTS IN NORMAL SERA

Experiment No.	Initial Counts*			6-Hr. Cultures			24-Hr. Cultures			48-Hr. Cultures		
	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts
1	24·0	75·0	1·0				15·0	64·0	21·0	9·0	61·0	30·0
							12·0	60·0	28·0	4·0	45·0	51·0
5	26·0	68·0	6·0	24·0	66·0	10·0	15·0	67·0	18·0	6·0	60·0	34·0
6	24·0	72·0	4·0							14·0	70·0	16·0
7	8·0	83·0	9·0				7·0	68·0	25·0	3·0	50·0	47·0
8	19·5	57·0	23·5				6·5	15·0	78·5	5·0	22·0	73·0
11	36·0	58·0	6·0							15·0	50·0	35·0
28	40·0	57·2	2·8				25·0	61·1	13·9	12·9	58·1	29·0
29	25·7	74·3	0·0							4·9	27·7	67·4
31	25·6	74·2	0·2							7·0	73·2	19·8
34	46·9	35·4	17·7	12·2	49·4	38·4	13·6	25·2	61·2			
35	26·3	68·4	5·3				20·0	60·0	20·0	6·3	49·4	44·3
43	31·5	63·0	5·5				15·2	71·7	13·1	7·0	70·0	23·0
45	17·8	64·4	17·8	13·0	67·0	20·0	13·0	43·5	43·5			
46	21·6	64·9	13·5	10·8	76·1	13·1	6·2	81·4	12·4	0·0	59·0	41·0
48	21·0	68·5	10·5	17·0	65·4	17·6	9·5	69·9	20·6	0·0	67·0	33·0
49	23·8	73·3	2·9	22·1	73·9	4·0	5·3	68·5	26·2	0·0	41·2	58·8
52	20·0	80·0	0·0	9·6	65·4	25·0	3·0	58·0	39·0			
54	14·4	85·6	0·0	21·6	72·2	6·2	11·0	82·2	6·8	0·0	75·0	25·0
57	18·2	81·8	0·0				15·4	72·3	12·3	4·5	42·2	53·3
64	15·1	80·6	4·3				15·9	57·9	26·2	12·0	45·1	42·9
71	13·0	86·0	1·0							2·0	48·0	50·0
73	18·0	73·0	9·0							7·0	55·0	38·0
										11·0	49·0	40·0
75	17·0	81·0	2·0							0·0	57·0	43·0
										1·0	26·0	73·0
Means ..	23·2	70·6	6·2	16·3	67·0	16·7	12·3	60·3	27·4	5·7	52·2	42·1

*Distribution of nucleated red cells in percentages.

cells and show only few mitotic figures decreases in culture (Table I and Fig. 1). The polychromatic normoblasts, initially about 70% of all the nucleated red cells, show numerous mitotic figures, but their number also decreases during 48 hours' culture. The number of orthochromatic normoblasts, on the other hand, which normally represent less than 10% of all the nucleated red cells, increases in the cultures greatly.

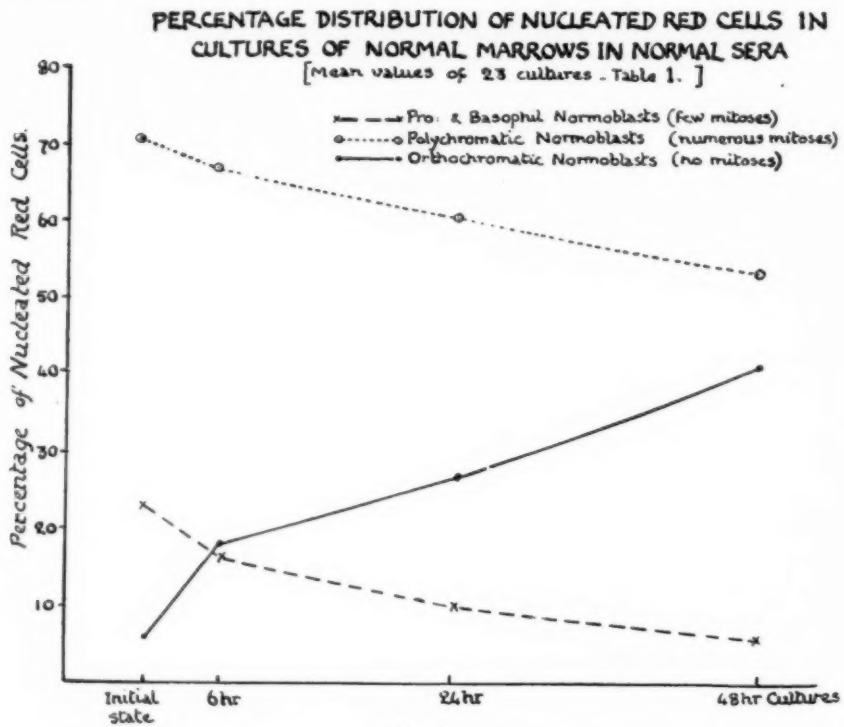


FIG. 1

No mitotic figures were seen in orthochromatic normoblasts, therefore their increase in number was attributed to a process of maturation from the earlier nucleated red cell forms. The maturation of polychromatic normoblasts not only gives rise to orthochromatic normoblasts but also to reticulocytes (polychromatic erythrocytes).

These results are confirmed by absolute nucleated cell counts also (Table II and Fig. 2) and show that maturation from earlier cell forms to later cell forms, similar to that observed *in vivo*, occurs in the cultures.

The appearance of mitotic figures on a slide is not necessarily a sign of multiplication, and to demonstrate the dual process of multiplication and maturation in the cultures an experiment was planned where the cell multiplication was inhibited by colchicine. Fig. 3 shows the results expressed in absolute numbers. Colchicine

in 1 : 10^6 concentration by inhibiting mitosis abolishes the increase of polychromatic normoblasts during the first six hours of the culture as well as the gradual increase in number of orthochromatic normoblasts. It produces also a sharper fall in the early red cell precursors. These results indicate that both cell proliferation and

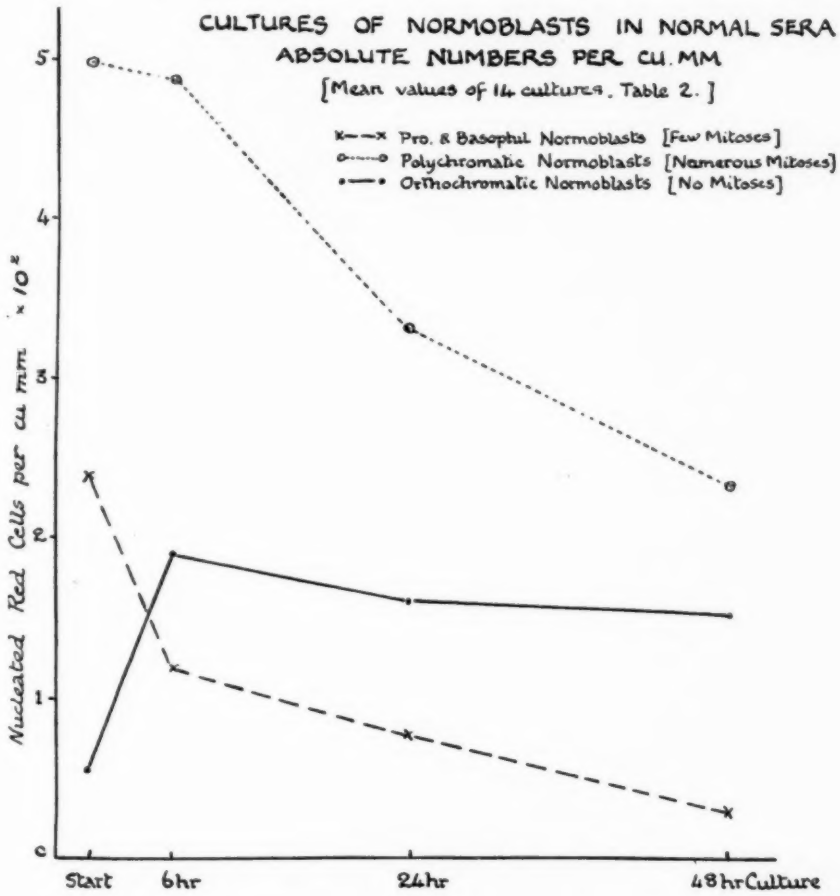


FIG. 2

differentiation occur in the cultures and that the increase in number of orthochromatic normoblasts is due to multiplication and maturation of the earlier cell forms. This concentration of colchicine was found not to be toxic for the cells, no increase in "cell death" (smear cells) was observed, and no appreciable effect on cell maturation was noted.

TABLE II

CULTURES OF NORMOBLASTS IN NORMAL SERA

Experiment No.	Initial Counts*			6-Hr. Cultures			24-Hr. Cultures			48-Hr. Cultures		
	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts	Pronormoblasts and Basophil Normoblasts	Polychromatic Normoblasts	Orthochromatic Normoblasts
28	448	640	32				279	682	155	120	540	270
29	180	520	0							20	113	274
31	196	570	2							36	375	101
34	1,384	1,044	522	352	1,434	1,115	240	448	1,088			
35	200	520	40				80	240	80	16	125	112
43	189	378	33				90	430	78	28	280	92
45	50	180	50	40	200	60	30	100	100			
46	120	360	75	87	612	105	32	416	64	0	165	115
48	40	130	20	28	108	29	6	44	13	0	20	10
49	130	400	16	90	300	16	16	210	80	0	80	114
52	20	80	0	10	68	26	5	87	58			
54	130	770	0	210	700	60	80	600	50	0	450	150
57	180	810	0				100	470	80	20	190	240
64	98	525	28				75	274	124	56	210	200
Means ..	240	498	58	117	489	187	78	333	164	27	232	152
Total of means		796			793			575			411	

* Absolute numbers per c.mm.

The changes in nucleated red cells only are dealt with in this paper since the current problems were concerned with erythropoiesis. No relevant changes were noted in the white cell series throughout the experiments. The white cells survive much longer in cultures than the red cells and maturation from early cell forms to late cell forms could be readily observed in all the cultures of both normoblastic and megaloblastic marrows.

In contrast to white cells nucleated red cells do not survive for many days in culture. After 48 hours most cultures show increasing degenerative changes. Less than one-half of the cultures showed a satisfactory level and morphology of nucleated red cells at 72 hours and only very occasional cultures at 96 hours. Because of this, most observations have been made in 24- to 48-hour cultures.

The conclusion drawn from these observations was that human bone marrows cultured *in vitro* approach the behaviour of the bone marrow observed *in vivo*. Two differences from the *in vivo* conditions, however, should be noted. The first is the rapid loss of proliferative power. After the first six to eight hours of culture the multiplication does not keep in step with the differentiation, resulting in a gradual accumulation of the late cell forms. This could be explained by a rapid exhaustion from the culture medium of a factor, or factors, necessary for cell proliferation, or by the absence *in vitro* of a cell type (? reticulum cell) responsible for the con-

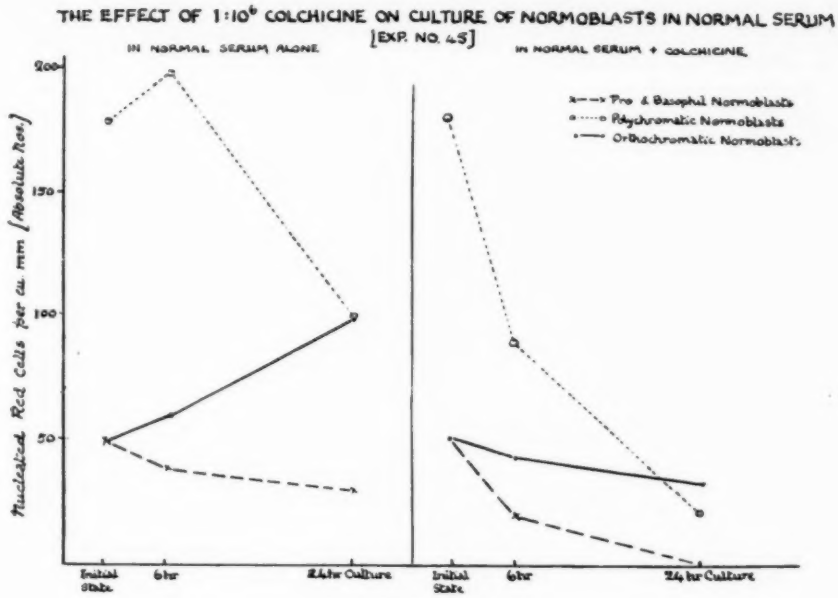


FIG. 3

tinuous production of early cell forms. Some evidence for the former theory is that embryo extract is necessary for maintaining multiplication in all tissue cultures. The use of such an extract with its many unknown factors was considered undesirable in these experiments.

The second difference in cultures from the *in vivo* behaviour of the normal bone marrow is the gradual accumulation of orthochromatic normoblasts. In normal human bone marrows these cells practically never exceed 10% of all the nucleated red cells. Indeed, an increase in their number is usually a sign of some disorder in red cell maturation. No satisfactory explanation can be given for their increase in number *in vitro*.

No tissue culture conditions are known which are identical with *in vivo* conditions, but it is reasonable to assume that what a cell or cell type can do *in vitro* it might also do *in vivo*. As the behaviour of 23 normal human bone marrows cultured in normal serum *in vitro* closely approached the behaviour of normal human bone marrows *in vivo*, it was concluded that the culture technique could yield useful information on the influence of different substances on erythropoiesis.

Culture of Megaloblastic Marrows *in vitro*

After the behaviour of normal human bone marrows *in vitro* had been assessed and the method of culture had been found satisfactory, similar studies on megalo-

blastic marrows were made. In all 25 megaloblastic marrows were cultured in a medium containing 35–80% serum (from normal subjects, or from patients with untreated pernicious anaemia) and 20–65% Gey or Ringer's solution.

The results presented in Table III indicate a considerable decrease in the percentage of the megaloblasts when cultured in normal serum as compared with the initial state. This was not unexpected as it was assumed that normal serum contains the factors necessary to normal cell maturation. The decrease, however, could have

TABLE III

CULTURES OF MEGALOBLASTS IN NORMAL AND PERNICIOUS ANAEMIA SERA*

Experiment No.	Initial Count	Cultures in Normal Serum (48 Hours)
B3	30·0	15·0
B4	23·5	3·5
B5	41·5	9·5
B6a	79·0	12·5
B6b	79·0	0·5
B8	18·5	1·5
B11	14·0	1·0
B13	32·5	4·5
B15	23·0	17·0
B16	35·0	7·0
B19	29·0	0·5
B22	29·5	2·5
B23	9·5	0·0
B27	11·5	1·0
B30	85·0	17·0
3	31·0	19·0
42	60·0	18·0
59	25·2	0·0
65	48·0	32·0
76	35·0	13·0
Means ..	37·0	8·8 (74·7% drop)

*Megaloblasts per 100 nucleated red cells. 35% serum + 65% Ringer's solution.

resulted from elective dying off of megaloblasts, from maturation of megaloblasts to megalocytes, or from conversion of megaloblasts to other cell forms. The examination of the cultures has shown that in the 48-hour cultures there was no increase in the number of smear cells, and no degenerative changes among the cells, but there were numerous transitional forms which suggested a transformation of megaloblasts into the normoblastic type of cell.

In experiments where absolute differential counts were made on the nucleated red cells it has been found that decrease in the number of megaloblasts in culture is accompanied by an increase in the number of normoblasts. This is clearly illustrated by a culture of a megaloblastic marrow in normal serum (Fig. 4). Ten similar experiments, included in Table IV and Fig. 5, demonstrate the same reciprocal changes. Here again the results are expressed in absolute numbers and show that whenever megaloblasts are cultured in a medium where a decrease in the numbers of

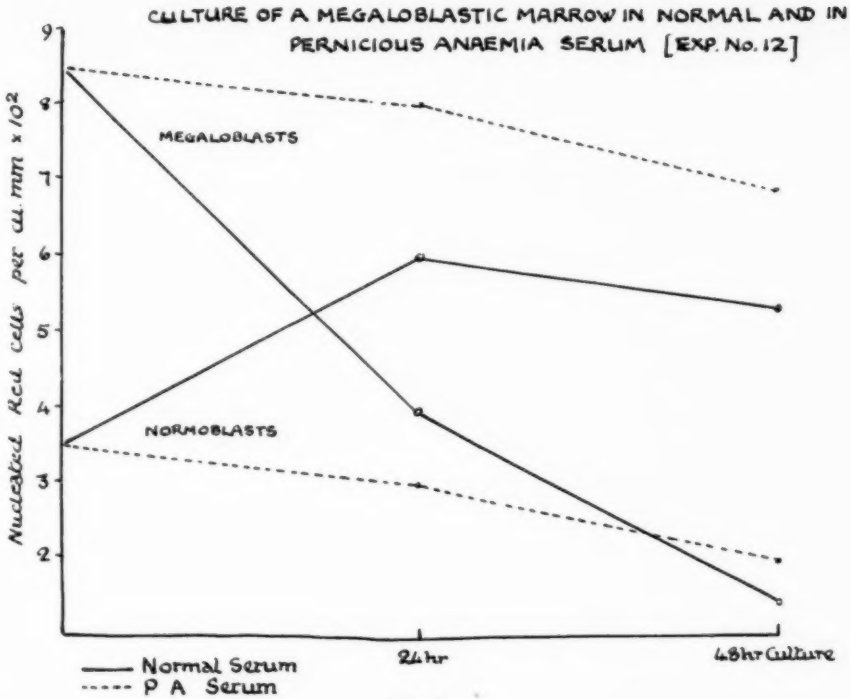


FIG. 4

megaloblasts not due to degenerative changes is noted, it is followed by a corresponding rise in normoblasts.

Fig. 6 illustrates this phenomenon further. Normoblasts from normal marrows when cultured in normal serum mature normally and their number gradually decreases during the culture. When megaloblastic marrows are cultured in normal serum the ripening of megaloblasts into normoblasts is such that the maturation of normoblasts is masked by it.

Further, that this increase in normoblasts is not an arrest of maturation but maturation to erythrocytes is shown by experiments in which reticulocytes were counted in cultures (Table V and Fig. 7). Reticulocytes ripen *in vitro* to erythrocytes and in cultures of peripheral blood the reticulocyte count decreases accordingly ; but when megaloblastic marrows are cultured in a medium in which large numbers of megaloblasts are transformed to normoblasts there is an increase in reticulocytes, i.e. the ripening of reticulocytes is masked by the production of new reticulocytes. This increase in reticulocytes is statistically significant.

These results indicate that when megaloblasts are cultured in normal serum their number decreases, this decrease being due to the transformation of megaloblasts to

TABLE IV

CULTURES OF MEGALOBLASTIC MARROWS IN NORMAL AND IN PERNICIOUS ANAEMIA SERA

Experiment No.	Initial Count*		Cultures in				Remarks
			Normal Serum		P.A. Serum		
	Megalo-blasts	Normo-blasts	Megalo-blasts	Normo-blasts	Megalo-blasts	Normo-blasts	
50	87	283	0	231			24 hr.
63	300	480	75	560			24 ,,
12	850	350	400	600	800	300	24 ,,
			150	550	700	200	48 ,,
B4	211	689	14	386	26	374	48 ,,
B5	456	644	71	679	115	585	48 ,,
B13	325	675	16	784	140	710	48 ,,
4	533	567	226	574	459	441	48 ,,
59	80	267	15	170	40	160	48 ,,
65	768	842	280	1,120	672	928	24 ,,
67	605	475	337	563	420	480	48 ,,
Means ..	423	527	144	565	374	464	
Total of means	950		709		838		

* Absolute numbers per c.mm.

TABLE V

RETICULOCYTE EXPERIMENTS

Source of Reticulocytes	Experiment No.*	Start†	Cultures			
			6-Hr.	12-Hr.	24-Hr.	48-Hr.
Peripheral blood	B9	2·1	2·0	3·0	1·3	1·0
	B10	7·7	7·5	5·0	6·0	2·5
	B12	12·0	13·6	11·0	10·6	6·3
	B14	11·3	13·0	12·0	8·4	3·3
	B17	21·8	22·7	23·5	20·7	16·4
	Mean	11·0	11·8	10·9	9·4	5·9
Pernicious anaemia marrows in pernicious anaemia sera	B10	9·0	9·1	9·9	11·6	11·0
	B12	16·3	11·5	12·6	14·2	13·0
	B14	11·6	12·3	11·5	9·0	10·9
	B17	12·0	10·1	12·3	10·0	11·9
	Mean	12·2	10·8	11·6	11·2	11·7
Pernicious anaemia marrows in normal sera	B10	9·0	8·0	9·0	11·8	10·1
	B12	16·3	12·8	17·9	18·7	21·0
	B14	11·6	13·9	12·2	15·0	17·5
	B17	12·0	12·9	15·3	13·0	18·5
	Mean	12·2	11·9	13·6	14·6	16·8

* 1,000 R.B.C.s counted. † Reticulocytes per 100 red cells.

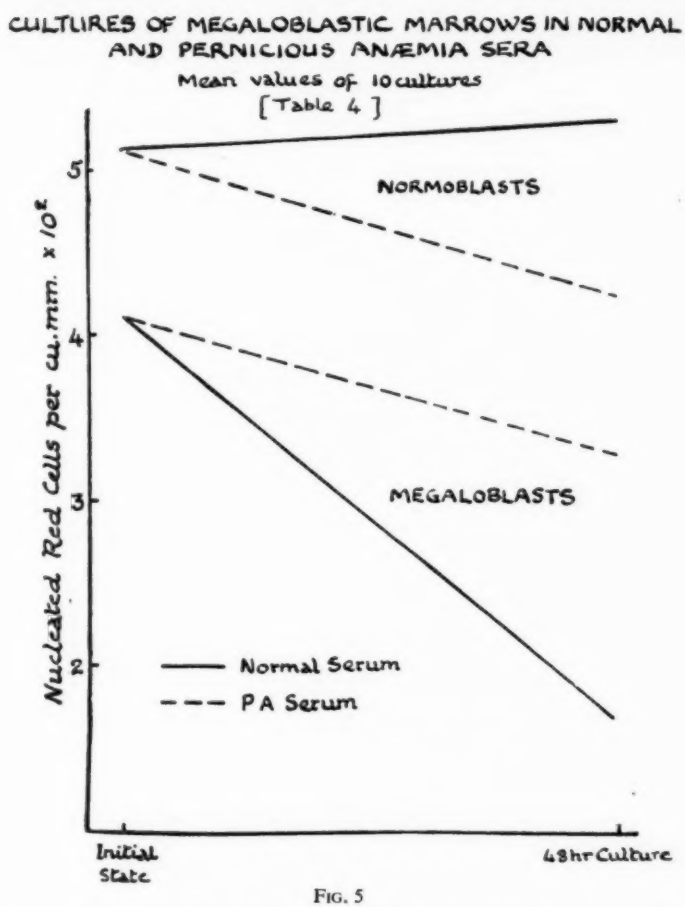


FIG. 5

normoblasts. This process may be so extensive as to mask the maturation of normoblasts to erythrocytes, and instead of a decrease in the numbers of normoblasts (which occurs when normoblastic marrows are cultured in normal serum) their number actually increases.

Transformation of Normoblasts into Megaloblasts

Experiments in which culture in undiluted pernicious anaemia serum caused transformation of originally normoblastic into megaloblastic marrows have been reported in a previous communication (Lajtha, 1950) and have been confirmed independently by Thompson (1951). It should be emphasized here that it was found necessary to keep the cultures incubated for 72 hours, since not all cultures

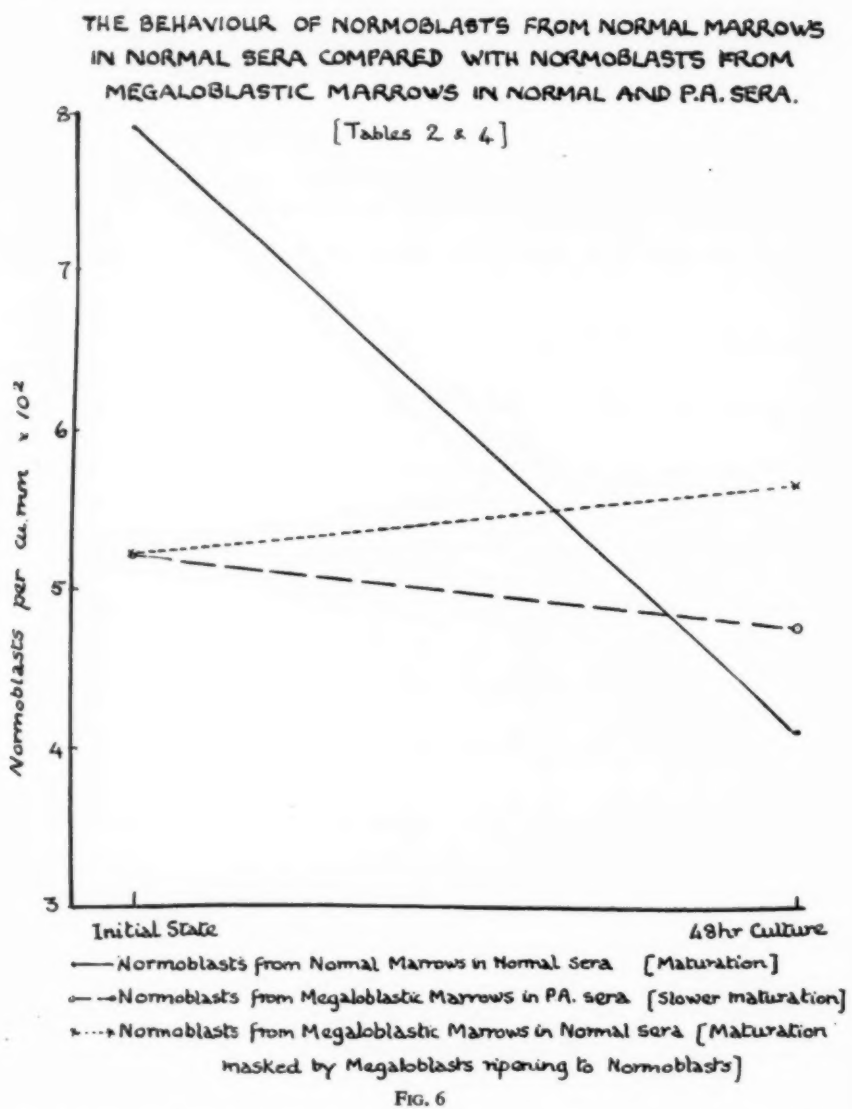

THE BEHAVIOUR OF NORMOBLASTS FROM NORMAL MARROWS IN NORMAL SERA COMPARED WITH NORMOBLASTS FROM MEGALOBLASTIC MARROWS IN NORMAL AND P.A. SERA.
[Tables 2 & 4]
Normoblasts per cu.mm x 10²
8
7
6
5
4
3
Initial State
48hr Culture
Normoblasts from Normal Marrows in Normal Sera [Maturation]
Normoblasts from Megaloblastic Marrows in P.A. sera [Slower maturation]
Normoblasts from Megaloblastic Marrows in Normal Sera [Maturation masked by Megaloblasts ripening to Normoblasts]

FIG. 6

develop megaloblasts in 48 hours. As normoblasts usually ripen considerably during 48 hours and their number decreases correspondingly, marrows with high erythropoietic activity were found to be the best sources for megaloblast production *in vitro*.

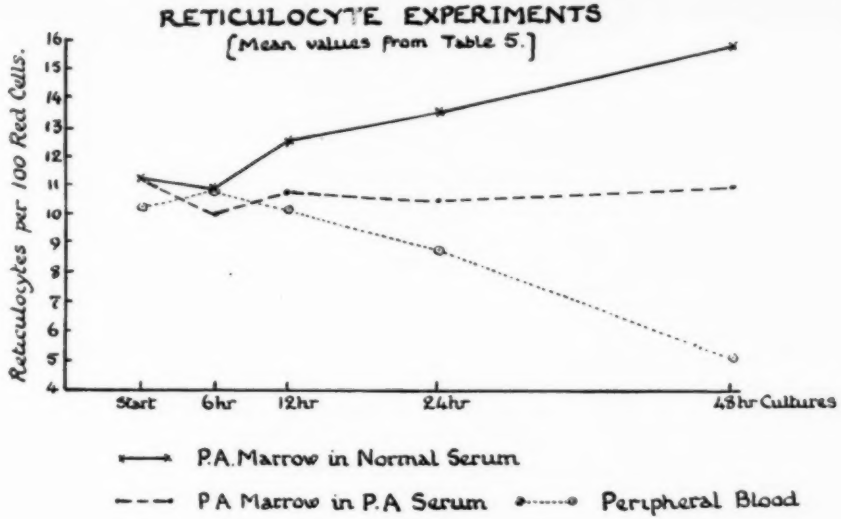


FIG. 7

The megaloblasts thus produced were not only morphologically classical megaloblasts, but they reacted to folic acid or vitamin B_{12} like those of megaloblastic marrows from pernicious anaemia patients (see photomicrographs).

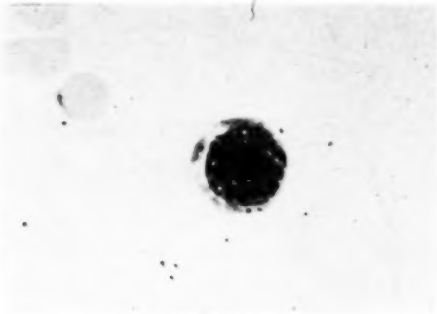
There is strong evidence that the megaloblasts of this "*in vitro* pernicious anaemia" were derived from normoblasts. Always numerous transitional "megaloid" forms from normoblasts to megaloblasts were seen, and there was no indication from the mitotic index that the megaloblasts (in one case 28%) had developed from a particular "young" cell form.

Discussion

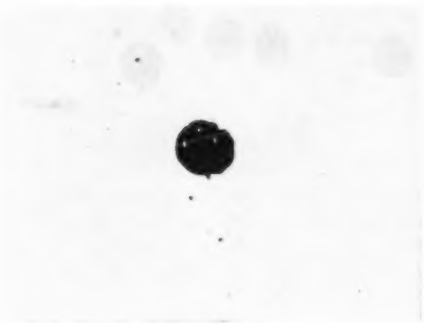
Attempts have been made to assess the maturation of the nucleated red cells by erythrocyte counting or haemoglobin determination. It was found, however, that none of the existing routine techniques are accurate enough to measure the small changes which occur *in vitro*. Theoretical calculation has shown that each nucleated red cell would have to produce at least 10 erythrocytes during the 48-hour culture to be able to demonstrate the phenomenon with erythrocyte counting or haemoglobin determination.

It has become evident during the experiments that the total nucleated cell count (which includes the "smear" cells) shows no appreciable changes during the first 24–48 hours of culture. On consideration this is not an unexpected observation because the aged and therefore dying white cells appear as "smear" cells and in the culture bottles there are not enough reticulo-endothelial cells to remove the dying cells. (In older cultures the lysis of the aged cells becomes more dominant and the absolute counts show a gradual decrease.) The maturation of the nucleated red cells to

TRANSFORMATION OF NORMOBLASTS INTO MEGALOBLASTS *in vitro* AND THE EFFECT OF FOLIC ACID AND VITAMIN B12

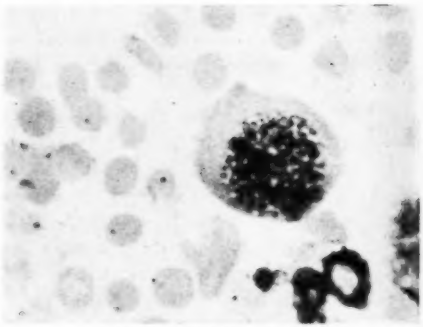


INITIAL STATE

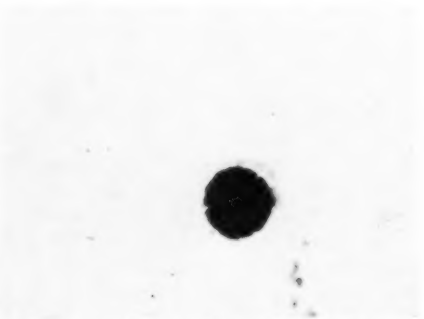


NORMAL SERUM

72-hour cultures in

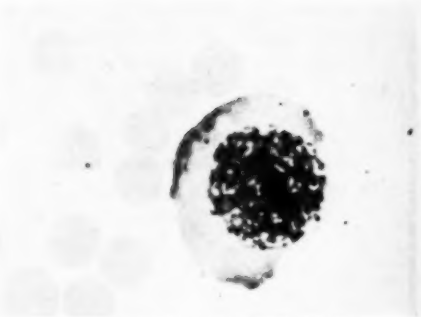


PERNICIOUS ANAEMIA SERUM



PERNICIOUS ANAEMIA SERUM + FOLIC ACID

72-hour cultures in



PERNICIOUS ANAEMIA SERUM + B12

erythrocytes, on the other hand, represents such a small change in the total nucleated cell counts that it is not always statistically evaluable. The changes, however, were always evident from the differential counts and in several experiments the results were obtained by differential counts only.

In using this marrow culture technique it is necessary to work out the personal error which depends on the total nucleated cell counts and the differential counts. With due care the error of total cell counts approaches the Poisson distribution; with considerable experience and conforming to very strict criteria it is possible to standardize the differential counts so as to approach the binomial distribution. This was in fact the case in the present experiments.

The normoblast-megaloblast interrelationship has been a matter of considerable controversy. The subject has been recently reviewed by Dacie and White (1949). Most workers now agree that transitional forms between the two series of cells do exist and that the megaloblast is a pathological form of normoblast. On the basis of uric acid excretion studies, Frühling has suggested that there is destruction of megaloblasts during response to therapy (Frühling, Mandel, Métais, and Moise, 1950). His findings, however, could equally well be explained by a rapid transformation of megaloblasts to normoblasts with subsequent loss of nuclei as these ripen to reticulocytes.

The observation that originally normoblastic marrows become megaloblastic when cultured in concentrated pernicious anaemia serum *in vitro*, and the finding that the majority of megaloblasts ripen to normoblasts (and not to megalocytes) when megaloblastic marrows are cultured in normal serum, are strong evidence for the reversibility between the normoblastic and megaloblastic series of cells. This reversibility could be observed at any stage of cell development, and implies that in the pernicious anaemia culture medium there is a change in the metabolism of the normoblasts which results, morphologically, in the megaloblastic form and, functionally, in a slower maturation.

Summary

A technique is detailed for the culture of human bone marrow *in vitro*.

Experiments suggesting a reversibility between normoblastic and megaloblastic series of cells are described.

I am greatly indebted to Dr. Rosemary Biggs for her help in the statistical evaluation of the error range of the technique, and to Mr. D. J. Finney for his invaluable help and advice in the assessment of the results of groups of experiments, and for his permission to quote his opinion.

References

Cairns, H. J. F., and Lajtha, L. G. (1948). *Nature, Lond.*, **162**, 536.
Callender, S. T., and Lajtha, L. G. (1951a). *Journal of Clinical Pathology*, **4**, 204.
—— —— (1951b). *Blood*, **6**, 1234.
Dacie, J. V., and White, J. C. (1949). *Journal of Clinical Pathology*, **2**, 1.
Ehrlich, P., and Lazarus, A. (1898). In *Specielle Pathologie und Therapie*, ed. Nothnagel, H., vol. 8. Wien.
Frühling, L., Mandel, P., Métais, P., and Moise, R. (1950). *Sang*, **21**, 541.
Gey, G. O., and Gey, M. K. (1936). *Amer. J. Cancer*, **27**, 45.
Jones, O. P. (1943). *Arch Path., Chicago*, **35**, 752.
—— (1947). *J. Lab. clin. Med.*, **32**, 700.
—— (1948). *Blood*, **3**, 967.

Lajtha, L. G. (1950). *Clin. Sci.*, **9**, 287.
Naegeli, O. (1931). *Blutkrankheiten und Blutdiagnostik*, 5th ed. Berlin.
Osgood, E. E., and Brownlee, I. E. (1937). *J. Amer. med. Ass.*, **108**, 1793.
Rusznyák, S., Löwinger, S., and Lajtha, L. G. (1948). *Hungarica Acta med.*, **1**, 9.
Thompson, R. B. (1951). Personal communication.

STATISTICAL APPENDIX

In Table VI s^2 is approximately equal to the means, and in Table VII $\frac{0.2558 \times 0.7442}{500} \times (100)^2$ is approximately equal to 4.8.

On the basis of statistical analysis of the error of the technique it is possible to assess, in each individual experiment, whether the differences noted are significant or not. Considerable difficulties are met, however, in attempting statistical analysis of groups of experiments. The difficulties arise because of the variability of the material. There is a wide range in the cellular composition of different bone marrows, and similarly a wide range of degree of megaloblastosis in the pernicious anaemia marrows. Thus the initial values for any cell type (orthochromatic normoblasts or megaloblasts) vary considerably from marrow to marrow. Also there are differences in the actions of different normal or different pernicious anaemia sera. The same normal marrow cultured in three different normal sera will behave slightly differently in each, and three different normal marrows cultured in the same normal serum will also behave slightly differently. This applies to megaloblastic marrows and pernicious anaemia sera also.

No appropriate formula could be found which would take into account this inherent variability in the cellular composition of different marrows and in the action of different sera, and would incorporate the external information of the analysis of the error range

TABLE VI

SUMMARY OF STATISTICS OF NUCLEATED CELL COUNTS IN CULTURES

Experiment		Mean	s^2 (random)	s Pipettes (%)	Total Error (%)
CL2	A	60·0	61·6	0	13·1
	B	68·7	113·9	0	15·53
CL4	A	40·0	28·0	0	13·2
	B	39·4	66·6	7·4	22·03
CL5	A	36·3	36·7	0	16·7
	B	39·9	46·7	4·9	23·2
				Total	103·7
				Mean error of one square ..	17·28

Error mean of 8 squares $= \frac{17{\cdot}28}{\sqrt{8}} = 6{\cdot}1\%$

Error mean of 16 squares $= \frac{17{\cdot}28}{4} = 4{\cdot}32\%$

In two experiments where eight squares were counted from each pipette the error found approximates this calculated error.

CL3	A	343·2	388·8	3·2	6·6
	B	330·1	807·7	0	8·6

TABLE VII

ERROR OF DIFFERENTIAL COUNTS*

Experiment No.	1st	2nd	3rd	4th	5th count	Total	Mean
Exp. B13 Slide 55	30·0	34·3	31·5	33·9	32·8	162·5	32·5
Exp. B16 Slide 67	10·1	13·5	14·7	12·9	14·0	65·2	13·0
Exp. B22 Slide 80	31·0	28·0	27·1	30·0	31·3	147·4	29·5
Exp. B22 Slide 84	25·1	23·0	30·1	30·3	28·0	136·5	27·3
Totals	96·2	98·8	103·4	107·1	106·1	511·6	

$Sx = 511{\cdot}6 \qquad \bar{X} = 25{\cdot}58$

$Sx^2 = 14282{\cdot}76$

$(Sx)^2 = 261734{\cdot}56$

$C.F. = \frac{(Sx)^2}{n} = 13086{\cdot}728$

SS total $= Sx^2 - CF = 1196{\cdot}03$

SS subject $= [(71016{\cdot}30)1/5] - CF = 1116{\cdot}53$

SS count $= [(52435{\cdot}06)1/4] - CF = 22{\cdot}03$

ANALYSIS OF VARIANCE

Source of Error	d.f.	SS	M.S.	s.
Total	19	1196·0		
Subject	3	1116·5	372·2	
Count	4	22·0	5·5	
Random	12	57·5	4·8	2·19

of the technique. A t test of the mean differences was in fact applied to the results of all the culture work to date. This confirmed the impression already formed of the significance of many of the results, but showed far less significance than was expected from sheer common sense, in view of the error range of the technique.

The advice of Mr. D. J. Finney, Lecturer in the Design and Analysis of Scientific Experiment, University of Oxford, was sought. His opinion was:

> "The magnitude of the difference between two types of culture varies from subject to subject. The information on errors examined strongly indicates that the difference is significant for each subject taken separately; the t test used on results of groups of experiments, however, would underestimate the degree of this significance because it employs a standard error calculated from the inter-subject variation in the difference and disregards the additional information on errors. Differences which are far outside the error range of the technique must be judged 'significant,' especially when large numbers of experiments on different subjects are taken into account, in the sense that they show clear evidence of some differential effect of type of culture, even though the size of the difference is not the same for all subjects."

J. clin. Path. (1952), **5**, 86.

THE BONE MARROW IN PAGET'S DISEASE (OSTEITIS DEFORMANS) WITH SPECIAL EMPHASIS ON THE MEGAKARYOCYTES

BY

PHILIP PIZZOLATO AND LOUIS RAIDER

From the Clinical Laboratory and Department of Radiology, Veterans Administration Hospital, and Department of Pathology, Charity Hospital of Louisiana, New Orleans, U.S.A.

(RECEIVED FOR PUBLICATION MAY 8, 1951)

In the course of a study of bone marrow in blood dyscrasias and other pathological states an increase of megakaryocytes was found to be rather frequent in idiopathic thrombocytopenic purpura, sickle cell anaemia, Hodgkin's disease, microcytic hypochromic anaemia, polycythaemia vera, and pregnancy. Simultaneously with this study, two patients with unexplained mild anaemia and no definite clinical diagnosis in whom sternal marrow examination showed an increase of megakaryocytes, and radiographs subsequently revealed Paget's disease of bone, were examined. A study was undertaken to investigate the sternal marrow of all available patients with monostotic and polyostotic forms of Paget's disease revealed radiologically.

Materials and Methods

Eight men at the Veterans Administration Hospital and two negro women from the Charity Hospital were studied. As controls, 10 patients were used who showed a megakaryocyte count in the marrow of 50 per c.mm. or above and had various blood dyscrasias. The sternal marrow (not more than 1 ml.) was aspirated with an 18 gauge spinal needle. A small quantity was used to make smears on slides and cover-slips. The remainder was added to 2 mg. of dry ammonium potassium oxalate (Heller and Paul, 1934). The oxalated marrow was diluted in a white cell pipette using Nile blue sulphate-aqueous propylene glycol (0.1% Nile blue in propylene glycol in equal parts of the glycol stain and distilled water at *p*H 6.5). The total nucleated elements were determined by means of a Levy-Neubauer chamber and the megakaryocytes were counted in a Fuch-Rosenthal chamber. With the Nile blue stain the megakaryocyte nucleus stained a deep blue and the cytoplasm, the periphery of which was often serrated, a clear or slightly granular blue. These cells measured from 20 to 50 μ. The other cells stained essentially the same, and the free fat stained a reddish orange.

The unused oxalated marrow was placed in a Wintrobe haematocrit tube and centrifuged to study the layer formation as fat, plasma, buffy coat, and red cell layer as recommended by Limarzi (1939). The buffy layer contained numerous megakaryocytes, and smears were prepared from this concentrated material. The smears were stained with Wright stain by the usual technique. It was found that the cover-slip gave a more uniform distribution of the giant cells as the slide method caused the accumulation of these cells in the feather-edge. One hundred megakaryocytes were counted and they

TABLE Ia

PERIPHERAL BLOOD AND MARROW FINDINGS IN PAGET'S DISEASE

Case No.	Bones Involved	Age	Colour	Sex	Peripheral Blood			
					Red Cells (per c.mm. in Millions)	White Cells (per c.mm. in Thousands)	Alkaline Phosphatase	Acid Phosphatase
1	Lumbosacral vertebrae ..	56	C.	F.	4·9	9·6	16·9	0·51
2	Lumbosacral vertebrae, pelvis, femurs	57	C.	F.	4·1	7·8	7·0	*
3	Femur and pelvis	59	C.	M.	3·6	12·3	5·1	0·26
4	Parietal	75	W.	M.	5·2	6·5	*	*
5	Lumbar vertebrae, and ilium	66	W.	M.	2·6	6·8	5·1	0·1
6	Humerus	50	W.	M.	5·1	6·5	12·4	0·22
7	Parietal, scapula, thoracic vertebrae	61	W.	M.	3·1	5·0	5·6	0·55
	No. 7 (two months later) ..				4·5	6·0	*	*
8	Parietal, humerus, lumbosacral vertebrae, and ilium	58	W.	M.	4·6	5·0	4·9	0·69
9	Lumbosacral vertebrae, pelvis, and femurs	66	C.	M.	4·3	4·2	17·6	0·80
10	Lumbosacral vertebrae and pelvis	60	C.	M.	4·9	12·7	8·5	0·14

TABLE Ib

PERIPHERAL BLOOD AND MARROW FINDINGS IN PAGET'S DISEASE

Case No.	Sternal Marrow														
	Differential														
	Marrow Count								Megakaryocyte Count						
	Fat (%)	Megakaryocytes (per c.mm.)	Nucleated Cells (per c.mm. in Thousands)	Granulocytes (%)	Erythroid (%)	Lymphoid (%)	Eosinophils (%)	Reticulo-endothelial Cells (%)	Megakaryoblast (%)	Promega-karyocyte (%)	Lymphoid Type (%)	Polykaryocyte (%)	Adult (%)	Degenerated Form (%)	Mitosis (%)
1	6	352	250	57·4	24·1	18·5	2·7	0·3	1	18	5	18	45	13	0
2	8	58	73	59·1	20·4	20·5	2·9	0·4	1	15	3	20	42	18	1
3	2	109	95	67·3	17·2	15·5	2·3	0·0	1	7	2	6	71	10	3
4	9	20	58	64·9	21·5	13·6	2·3	0·2	4	24	12	10	32	18	0
5	3	12	58	70·3	16·5	13·2	3·4	0·6	4	10	8	10	54	14	0
6	5	11	27	66·5	25·5	18·0	3·6	0·1	1	16	5	3	62	12	1
7	*	104	89	54·3	17·4	28·3	2·6	0·0	0	10	2	35	46	6	1
	3	53	50	57·5	15·1	27·4	5·3	0·4	2	6	2	9	65	16	0
8	9	59	112	59·7	22·8	17·5	1·5	0·0	3	14	8	7	51	16	1
9	7	66	48	47·6	29·4	23·0	1·9	0·0	0	9	4	9	66	11	1
10	6	40	84	63·0	23·7	13·3	2·3	0·0	3	7	9	10	55	15	1

* Not done.

were divided, with some modification, into the classification as recommended by Dameshek and Miller (1946) as megakaryoblast, lymphoid types, polykaryocytes, adult or multilobulated forms, degenerated varieties, and mitotic forms.

A differential count of 1,000 marrow cells on each patient with osteitis deformans was done, the cells being classified as granulocyte, erythroid, and lymphoid elements, eosinophils, and reticulo-endothelial cells (Table I). Erythrocyte and leukocyte counts of peripheral blood were recorded on patients with Paget's disease and on the control group. Serum acid and alkaline phosphatase estimations were performed on patients with Paget's disease and recorded in Shinowara-Jones-Reinhart units (normal, 0–1.1) and Bodansky units (normal, 2–9).

A differential megakaryocyte count was done on 10 patients with no purpuric or thrombotic tendencies as a control group ; patients were selected who had megakaryocyte counts above 50 per c.mm. (Table II).

TABLE II

PERIPHERAL BLOOD AND MARROW FINDINGS IN PATIENTS WITH ELEVATED MEGAKARYOCYTE VALUES

Disease	Colour	Sex	Age	Peripheral Blood		Sternal Marrow									
									Megakaryocyte Differential Count						
				Red Cells (per c.mm. in Millions)	White Cells (per c.mm. in Thousands)	Fat (%)	Megakaryocyte (per c.mm.)	Nucleated Cells (per c.mm. in Thousands)	Megakaryoblast (%)	Promegakaryocyte (%)	Lymphoid Type (%)	Polykaryocyte (%)	Adult (%)	Degenerated Form (%)	Mitosis (%)
Acute haemolytic anaemia	W.	M.	20	3·7	10·8	0	281	300	1	7	5	10	70	7	0
Carcinoma of stomach	W.	M.	61	3·2	19·6	10	156	303	2	10	5	8	55	19	1
Sickle cell anaemia ..	C.	F.	25	1·8	9·0	0	153	275	1	4	1	8	75	11	0
Polycythaemia vera ..	C.	M.	33	5·8	8·3	2	50	95	1	5	8	0	73	13	0
Thiouracil neutropenia ..	W.	M.	26	4·6	5·9	4	73	89	0	13	6	7	58	16	0
Macrocytic anaemia ..	W.	M.	64	4·2	8·5	9	73	190	3	11	5	11	45	24	1
Leukocytosis, unknown cause ..	W.	M.	50	5·1	15·1	*	75	106	1	7	14	6	59	12	1
Macrocytic anaemia ..	W.	M.	56	3·8	8·3	8	109	119	0	8	4	5	76	7	0
Hodgkin's disease	W.	M.	50	4·5	6·0	14	128	127	1	6	4	8	68	13	0
Multiple sclerosis	W.	M.	32	3·1	4·4	2	52	132	1	6	5	3	77	7	1

* Not done.

The possible relationship between the marrow megakaryocytes and the serum acid and alkaline phosphatase levels was studied on 11 patients with no obvious bone pathology (Table III).

TABLE III

MEGAKARYOCYTES AND SERUM ACID AND ALKALINE PHOSPHATASE LEVELS IN PATIENTS WITH NO BONE PATHOLOGY

Megakaryocytes (per c.mm.)	Phosphatase	
	Acid	Alkaline
36	0·18	4·2
61	0·26	2·9
72	0·14	1·3
72	0·18	3·0
45	0·25	5·5
18	0·55	5·1
56	0·28	7·1
1	0·36	5·6
65	0·73	5·2
167	1·40	4·5
12	0·58	3·6

The diagnosis of osteitis deformans was made by radiographs of the bone showing the characteristic coarse trabeculations and cortical thickening. In one case of pathological fracture a biopsy of the fracture site at operation revealed Paget's disease (Case 6). As interpretation of radiological findings in the sternum was extremely difficult, no special technique was used in the detection of lesions in the sternum.

Comment

According to Jaffe (1933) the marrow in the typical picture of Paget's disease shows classical osteoclasts and osteoblasts with polymorphonuclear leukocytes, many of which are eosinophils, and varying numbers of haemosiderin-containing macrophages. There may be fatty marrow with islands of myeloid tissue. There may be areas of fibrous marrow which in the process of healing may become replaced by more lymphoid and fatty marrow. Since the Italian school of haematology, notably Di Guglielmo (1925), believes that the multinucleated megakaryocyte (polykaryocyte) is probably identical with the osteoclast and that the multilobulated forms may represent developmental stages in platelet formation, the increase of all forms of these giant cells may be significant in the pre-radiological evidence of osseous involvement. One feature in most of our cases was the increase of megakaryocytes out of proportion to the activity of the marrow. In a study of 10 normal individuals (Pizzolato, 1948) the average number of megakaryocytes was 13 per c.mm., with one individual having 22 per c.mm. as the upper limit. Three individuals with osteitis deformans had counts within the normal limits. One patient had a value of 352 per c.mm., the highest recorded in any condition. It is to be noted that the relative number of multinucleated forms was not increased ; in only two cases were counts of 18% and 20% found recorded. These varieties may be more frequent, however, due to the fact that nuclei are often superimposed on each other, giving the impression of adult or multilobulated forms. Again, there are instances where platelets cover the nucleus. Nearly all megakaryocytes appear to have the capacity for platelet formation, and the platelets of the marrow and peripheral blood have normal morphological and chromatic characteristics ; thrombotic phenomena do not occur.

In the control series an increase of megakaryocytes was associated with other activity in the bone marrow, such as anaemia or leukopenia. Only two individuals with Paget's disease showed hyperplasia of the marrow compared with the maximum normal nucleated elements of 105,000 per c.mm.

No correlation between the marrow megakaryocytes and the serum phosphatase levels was observed.

Eosinophils were not increased: the maximum was 5.2% compared with the classic study of Segerdahl (1935) on 18 persons above 66 years of age; her findings were 3.25%. Although various methods gave slightly different results, the values obtained did not suggest an eosinophilia (Pizzolato, 1949). The reticulo-endothelial cells or macrophages, as Jaffe refers to them, ranged from 0.0% to 0.6% and are not significantly different from Segerdahl's average of 0.09%.

Jaffe also frequently observed a fatty marrow. Fat, as encountered in the upper layer of the haematocrit, was increased from 5 to 9% in seven of the 10 instances compared with the normal of from 1% to 3%. The significance of this excess fat in the presence of a normal or slightly hyperplastic marrow we are not prepared to state.

Summary

In the presence of suspicious radiological findings in bone, a study of the bone marrow should be undertaken. Sternal marrow aspirated from 10 patients with osteitis deformans showed an increase in the number of megakaryocytes and in the fat as observed in the haematocrit tube. The remainder of the marrow was essentially normal.

Sponsored by the Veterans Administration and published with the approval of the chief medical director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

References

Dameshek, W., and Miller, E. B. (1946). *Blood*, **1**, 27.
Di Guglielmo, G. (1925). *Haematologica*, **6**, 156.
Heller, V. G., and Paul, H. (1934). *J. Lab. clin. Med.*, **19**, 777.
Jaffe, H. L. (1933). *Arch. Path.*, **15**, 83.
Limarzi, L. R. (1939). *Illinois med. J.*, **75**, 38.
Pizzolato, P. (1948). *Amer. J. clin. Path.*, **18**, 891.
—— (1949). *Amer. J. med. Technol.*, **15**, 213.
Segerdahl, E. (1935). *Acta med. Scand.*, Suppl. 64.

J. clin. Path. (1952), **5**, 91.

THE EFFECTS OF A PHENOTHIAZINE DERIVATIVE (RP.3300) ON RED CELL PRESERVATION

BY

H. CHAPLIN, JUN.,* HAL CRAWFORD, MARIE CUTBUSH, AND P. L. MOLLISON

From the Medical Research Council Blood Transfusion Research Unit, Postgraduate Medical School of London

(RECEIVED FOR PUBLICATION JUNE 30, 1951)

In the course of a search for anti-histamine compounds Halpern (1947) found that a new group of drugs, derived from phenothiazine (thio-diphenyl-amine), was very active. In the following year Halpern and Hamburger (1948) gave a more detailed account of the effects of one of these derivatives, to which they gave the name "phenergan." It was subsequently reported that the phenothiazine derivatives possessed extraordinary power as histamine antagonists far exceeding that of previously described anti-histamine compounds which lacked the diphenyl radical but possessed the basic formula $R—N—(CH_2)_x—N\begin{matrix}CH_3\\CH_3\end{matrix}$ (Halpern, 1950).

Halpern and Reuse (1949), working with these compounds on isolated animal tissues, made the interesting observation that frog muscle suspended in distilled water to which phenergan had been added failed to show the rapid tissue lysis observed in distilled water alone. In an isotonic solution containing phenergan tissues retained their normal histological appearance for periods of from 30 to 120 days. These studies were extended to other tissues (skin, liver, testicle, etc.) and similar effects were observed.

Halpern, Dreyfus, and Bourdon (1950) later studied the effects of these compounds on blood during storage. Blood was drawn into Wurmser's (1946) solution (citric acid, 3 g., sodium hydroxide, 1 g., water, 100 g., added to 400 ml. blood) and stored at 5° C. for varying periods. Phenergan or the related compound RP.3300 was added to the Wurmser's solution to achieve final concentrations in the stored blood mixture of 1 in 10,000 and 1 in 25,000. In the blood samples preserved in the presence of phenothiazine derivatives it was found that (*a*) the time of appearance of spontaneous haemolysis on storage was much delayed compared with the control samples ; (*b*) the normal sedimentation of the cells following mixing was maintained far longer than in the control samples ; (*c*) the normal tendency to rouleaux formation was maintained longer than in control samples ; (*d*) the shape and volume of red cells (both in suspension and as judged in stained smears) remained normal for a longer time than in control samples ; and (*e*) the rise in the serum potassium level during

* U.S. Public Health Service Research Fellow of the National Institute of Health.

storage was both delayed and decreased compared with control samples. Furthermore, Halpern and Bessis (1950) using electron and phase microscopy showed that red cells rendered spherical by repeated washing in physiological saline could be restored to their former biconcave shape by weak dilutions of phenothiazine derivatives.

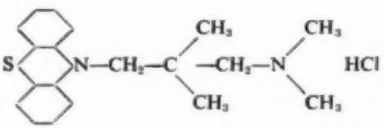
It was pointed out that there was no correlation between the anti-histaminic potency of a given phenothiazine derivative and its ability to evoke these favourable changes *in vitro* in stored blood. For example, RP.3300 has almost no anti-histaminic activity, yet has the most marked effect upon stored blood, whereas " neo-antergan " is strongly anti-histaminic, but has almost no effect on stored blood. Halpern suggested that the activity of these agents as tissue preservatives was most likely to be due to their power to inhibit enzymes, particularly cathepsins and tryptic enzymes.

Observations on white blood cells and platelets were less conclusive but, at least in so far as white cell staining and the resistance of white cells to spontaneous lysis during storage were concerned, the phenothiazine compounds seemed to exert a favourable effect. Preliminary transfusion experiments suggested that red cells stored with RP.3300 survived better *in vivo* than control red cells ; however, only two or three experiments were performed.

In view of this encouraging report it was decided to try to confirm the findings of Halpern and his colleagues and, particularly, to carry out a more detailed study of the survival in human subjects of red cells stored with RP.3300.

The Compound and Animal Toxicity Tests

The Compound.—RP.3300 is a white crystalline powder ; its hydrochloride has a molecular weight of 348.5 and has the following chemical structure :



The compound is moderately soluble in distilled water, but a precipitate forms if the *p*H is raised above 7, for example, by adding trisodium citrate.

A concentration of 1 in 1,000 of RP.3300 in distilled water lowers the surface tension of the water by 17 dynes (from 71) ; a concentration of 1 in 10,000 lowers the surface tension of water by 13 dynes.*

The compound in solution is relatively stable but, due to spontaneous oxidation, loses some of its activity after prolonged storage.

Toxicity Tests.—Since no information was available about the toxicity of the compound in man and there was only limited information about its toxicity in animals, the compound was submitted to Dr. Paton, of the National Institute for Medical Research. He found that in the chloralosed cat, RP.3300 in an intravenous dose of 1 mg./kg. caused a fall in blood pressure of 15 mm. Hg lasting only one to two minutes ; a dose of 4 mg./kg. produced a fall of 60 mm. Hg. The fall in pressure was shown to be due, in

* The authors are indebted to Dr. W. D. M. Paton, of the National Institute for Medical Research, Mill Hill, London, for the measurement of the surface activity of RP.3300 and for the toxicity experiments on animals mentioned below.

part at least, to a direct effect on the heart rather than to peripheral vasodilatation, since during the greater part of the depressor response limb volume decreased. This action of the drug was found to have a short latency, to resist atropine and " neo-antergan," and to be unaffected by vagotomy.

Dr. Paton suggested that the direct effect on the heart might well be due to the surface activity of the compound.

Respiration was unaffected by an intravenous dose of 1 mg./kg., but was profoundly, though transiently, depressed by 4 mg./kg.; this effect was abolished by cutting the vagi.

In the mouse the LD50 of the drug was found to be between 20 and 50 mg./kg.

Transfusion Experiments

In view of the results of the tests in animals, it was considered safe to transfuse blood containing a total dose of approximately 1–2 mg. RP.3300 per kg. of the patient's body weight, administered slowly (in approximately one hour). The patient's pulse, respiration rate, and blood pressure were recorded at intervals during the transfusion and for 15 minutes afterwards.

The method of preparing the preservative solution was as follows: 50 mg. of RP.3300 dissolved in 5 ml. of distilled water were added to 120 ml. of the standard acid-citrate-dextrose solution and autoclaved immediately. A few days later 420 ml. of blood were drawn into the bottle and then stored at approximately +2° C. for periods ranging from 20 to 35 days. Blood was also drawn into the standard anticoagulant acid-citrate-dextrose solution, to which no RP.3300 had been added. These control bottles were taken at the same time as the test bottles and stored with them in the same refrigerator until the time of transfusion.

Two types of experiment were performed. In the first type, " single population," three recipients (group O, type M) each received approximately 500 ml. of a concentrated red cell suspension prepared from two bottles of group O, type N, blood of identical age, stored with RP.3300. In these cases the transfusion was completed in 30 to 70 minutes and a sample was taken from the patient five minutes after transfusion. The concentration of transfused red cells in this sample was taken as 100% survival; the counts of surviving red cells in subsequent samples were expressed as percentages of this initial figure. The resulting estimates of survival could be compared with earlier estimates made by the same method for red cells stored with acid-citrate-dextrose alone (Loutit and Mollison, 1943).

In the second type of experiment, which may conveniently be referred to as " double population " experiments, the patient received a transfusion of red cells stored without RP.3300, as well as a transfusion of red cells stored with RP.3300. Six experiments of this type were performed. In five cases the recipient belonged to group A, type M, and received group A, type N, cells stored with RP.3300, and group O, type M, cells stored without RP.3300. In post-transfusion samples the AN cells were counted after differential agglutination with anti-M, and the OM cells after differential agglutination with anti-A. The sixth recipient was group A, Rh positive, and received group A, Rh negative cells (stored with RP.3300) and group O, Rh positive cells (controls): in this case an anti-Rh serum was used instead of anti-M in counting the surviving A cells.

On the day of transfusion four bottles of blood of identical age were selected, two having been stored with RP.3300 and two without. The supernatant plasma was withdrawn from each bottle and the red cells were then pooled, in pairs, to provide approximately 500 ml. of " test " red cell suspension (stored with RP.3300) and a similar quantity of control red cell suspension (stored without RP.3300). Now 10 ml. of a solution of A and B substances (Sharp and Dohme) were added to the concentrated suspension of group O cells. The two bottles of red cell suspension were transfused in succession in the course of one to two hours.

Before transfusion each bottle was well mixed and a sample was then withdrawn for red cell counting. The number of red cells counted (for each bottle) was between 2,000 and 3,000 in four cases, and between 4,000 and 6,000 in the remaining two cases. The volume of suspension in each bottle was measured; any residue in the bottle or apparatus at the end of transfusion was also measured and subtracted from the initial total. It was thus possible to estimate, within the limits of the error of the method, the expected ratio of the two types of red cell in the recipient's blood stream, assuming that the two types of red cell survived equally well. Any striking beneficial or harmful effect of RP.3300 could be detected by a deviation of the ratio found in the recipient's circulation at intervals after transfusion, from the ratio expected.

The technique of differential agglutination was that described by Dacie and Mollison (1943). However, for anti-M counts powdered dry globulin was added directly to a 1 in 100 red cell suspension (Ebert and Emerson, 1946). When anti-A and anti-M counts were performed on the same blood sample, the 1 in 100 suspension for the anti-M counts was prepared from the 1 in 50 suspension used for the anti-A counts, to minimize dilution errors.

The number of cells counted was never less than 2,000, and two-thirds of the estimates were based on counts of more than 4,000 cells.

Experiments *in vitro*

Acid-citrate-dextrose, 1 ml., or of disodium citrate solution, containing the required concentration of RP.3300, was placed in a clean, glass, capped bottle and autoclaved for 20 minutes at 20 lb./sq. in. pressure at 121° C. Similar bottles containing the anti-coagulant solution alone were prepared for control samples. Then 4 ml. of whole heparinized blood were added to each bottle, with sterile precautions. Blood samples were stored either at 4° C. or at 37° C.

Haemoglobin Concentrations.—These were determined as oxyhaemoglobin using a photoelectric colorimeter.

Haematocrits.—Haematocrits were determined in Wintrobe tubes centrifuged at a radius of 15 cm. for 30 minutes at 3,000 r.p.m.

Osmotic Fragility.—This was determined by the method of Parpart, Lorenz, Parpart, Gregg, and Chase (1947), but omitting the addition of complementary solutions. Forty-five minutes after adding the red cells to the test solution the tubes were centrifuged at 3,000 r.p.m. for five minutes, and the supernatants withdrawn for colorimetric estimation.

Free Haemoglobin in the Plasma.—This was determined by a modification of the benzidine method of Bing and Baker (1931).

Plasma Potassium.—Determinations were made with a flame photometer (Domingo and Klyne, 1949).

RP.3300.—Determination in plasma was performed by an ether-extraction sulphuric acid oxidation method (Halpern, 1951).

Results

Clinical Observations.—No changes in blood pressure, respiration, or pulse rate were observed after transfusion and no unusual symptoms were noted. One of the nine recipients developed a febrile reaction, but this did not differ in any way from the ordinary type of febrile reaction due to pyrogens.

Survival of Transfused Red Cells in "Single Population" Experiments.—The three recipients in this series received red cells stored with RP.3300 for 23, 27, and 28 days respectively. Estimates made at 24 hours showed that survival did

not differ significantly from that of red cells stored in acid-citrate-dextrose alone (Table I). Estimates made at 48 hours confirmed these observations. These cases were not followed further.

TABLE I

THE SURVIVAL OF RED CELLS STORED WITH RP.3300 ("SINGLE POPULATION" EXPERIMENTS)

		No. of Cases	Preservative Solution	Time of Storage (Days)	Percentage[1] Survival at 24 Hrs.
Loutit and Mollison (1943)		2	A.C.D.[2]	20	92
		7	A.C.D.	28	81
Present Investigators	1.	1	A.C.D. + RP.3300[3]	23	89
	2.	1	A.C.D. + RP.3300	27	91
	3.	1	A.C.D. + RP.3300	28	67

[1] The concentration of surviving red cells in the sample obtained immediately after transfusion was taken as "100% survival."
[2] Acid-citrate-dextrose.
[3] RP.3300 added to give a final concentration in the blood-anticoagulant mixture of 1/10,000.

In "Double Population" Experiments.—For the sake of simplicity the expected ratio, based on the numbers of the two types of red cell transfused, has in each of the six cases been converted to unity, and the ratios found in the recipient's circulation have been multiplied by the same factors.

For example, Mrs. T. was one of the two patients who received blood stored for 27 days (see Fig. 1). A concentrated suspension of red cells, 477 ml., count 7.19×10^6/c.mm., stored with RP.3300, was first transfused, followed by 534 ml. of a suspension of red cells, count 6.98×10^6/c.mm., stored in acid-citrate-dextrose alone. (The red cell estimates were based on counts of 4,000 cells.) Thus the expected ratio of the two types of red cell in the patient's circulation after transfusion was:

$$\frac{477 \times 7{\cdot}19}{534 \times 6{\cdot}98} = 0{\cdot}92$$

Estimates made on the following morning gave the following results: A cells ("test" population) 0.611×10^6/c.mm.; O cells ("control" population) 0.747×10^6/c.mm. (The estimates were based on counts of approximately 6,000 cells; in each case a pre-transfusion blank of 10,000 unagglutinated cells per c.mm. was subtracted from the observed count.) The ratio was thus

$$\frac{611}{747} = 0{\cdot}818$$

Converting the ratio expected to 1.0, the ratio found was

$$0{\cdot}818 \times \frac{1{\cdot}0}{0{\cdot}92} = 0{\cdot}89,$$

and this is the ratio entered on Fig. 1. Thus a ratio of

$$\frac{\text{survival of test cells}}{\text{survival of control cells}}$$

which is less than 1 indicates an adverse effect of RP.3300, and a ratio greater than 1 a favourable effect. The results in the six cases are displayed graphically in Fig. 1. Twenty-four hours after transfusion survival is better than expected in two cases, but worse than expected in four cases. In five cases examined between 40 and 60 days after transfusion survival is as good as that of the control in one case, but is worse than that of the control in the remaining four cases.

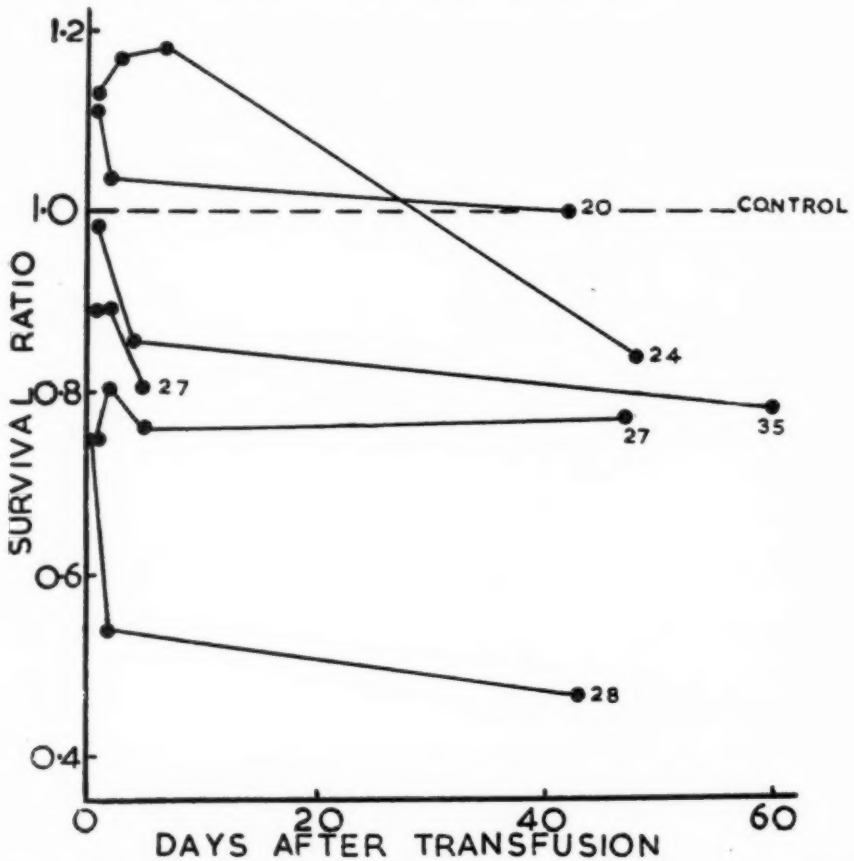


FIG. 1.—The post-transfusion survival of red cells stored in acid-citrate-dextrose with RP.3300 compared with that of red cells stored in acid-citrate-dextrose alone (control). The figures at the end of each curve indicate the previous length of storage in days.

Amount of RP.3300 in the Blood after Storage.—In view of a personal communication from Halpern that RP.3300 oxidizes on storage, it was considered desirable to demonstrate that blood which had been stored with RP.3300 for several weeks still contained an adequate amount of the compound.

Accordingly the supernatant plasma-diluent from a bottle of blood stored for 35 days was examined ; the concentration of RP.3300 was found to be approximately 1 in 12,000. (The original concentration in the mixture of blood and diluent was 1 in 10,000 ; assuming that no RP.3300 entered the red cells, the concentration in the plasma-diluent may originally have been as high as 1 in 7,000.) The observed concentration is within the range which had previously been considered optimal for storage and certainly within the range known to produce "beneficial" effects *in vitro*.

Spontaneous Haemolysis on Storage.—Blood to which RP.3300 had been added to give a final concentration of 1 in 10,000 haemolysed less rapidly than did control samples, whether stored at 37° C. or 4° C. RP.3300 in a final concentration of 1 in 20,000 produced slightly less effect ; a final concentration of 1 in 5,000 produced immediate haemolysis of varying degrees. Some typical results are set out in Tables II and III.

The diminution in the rate of spontaneous haemolysis produced by RP.3300 was very obvious in the whole bottles of blood used for transfusion. Whereas the super-

TABLE II

PLASMA HAEMOGLOBIN BEFORE AND AFTER STORAGE at 4° C. IN A.C.D. WITH AND WITHOUT RP.3300

Blood Sample	Plasma Haemoglobin (mg. per 100 ml.)			
	Before Storage		After 1 Month	
	With RP.3300*	Without RP.3300	With RP.3300	Without RP.3300
H.C.	3	4	80	188
P.L.M.	3	4	65	148
M.C.	4	4	74	151

* RP.3300 was used in a final dilution of 1 in 10,000. Value given = mean of two or more samples.

TABLE III

PLASMA HAEMOGLOBIN BEFORE AND AFTER STORAGE AT 4° C. IN DISODIUM CITRATE WITH AND WITHOUT RP.3300

Blood Sample	Plasma Haemoglobin (mg. per 100 ml.)					
	Before Storage[1]			After 14 Days[2]		
	RP.3300 (1/10,000)	RP.3300 (1/20,000)	Without RP.3300	RP.3300 (1/10,000)	RP.3300 (1/20,000)	Without RP.3300
H.C.J. ..	9	6	4	43	64	75
P.L.M. ..	7	7	5	17	27	64
H.C. ..	11	9	6	23	30	83

[1] Single samples. [2] Mean of two samples.

natant plasma of the control bottles, stored without RP.3300 for 20 to 35 days, was usually tinged with free haemoglobin, that of the bottles stored with RP.3300 for the same period was always a light yellow, free from any trace of pink.

Osmotic Fragility.—Blood samples containing RP.3300, stored at 4° C. or at 37° C., showed an increased resistance to hypotonic saline ; an example is given in Table IV. Again, RP.3300 in a final concentration of 1 in 20,000 had less effect than a final concentration of 1 in 10,000.

TABLE IV

PERCENTAGE HAEMOLYSIS IN 0·55% BUFFERED SALINE AFTER ONE MONTH'S STORAGE AT 4° C. IN A.C.D. WITH AND WITHOUT RP.3300 IN A FINAL DILUTION OF 1 IN 10,000

Blood Sample	Percentage Haemolysis	
	With RP.3300	Without RP.3300
H.C.	15	43
M.C.	4	37
P.L.M.	6	28

It was observed that fresh red cells added to hypotonic saline solutions to which RP.3300 had been added to a final concentration of 1 in 40,000 showed less haemolysis than in a control solution containing hypotonic saline alone. In order to balance the effect on tonicity and *p*H produced by the small amount of RP.3300 solution added, an equal volume of an equimolar solution of ammonium chloride was added to the control.

As shown in Table V red cells added to the test solution showed strikingly less haemolysis than in the controls. RP.3300 appeared to exert this effect even in a final concentration of 1 in 160,000.

TABLE V

PERCENTAGE HAEMOLYSIS IN 0·45% BUFFERED SALINE WITH RP.3300 ADDED IN VARIOUS CONCENTRATIONS

1/20,000	3
Control*	66
1/40,000	9
Control	53
1/80,000	19
Control	47
1/160,000	32
Control	47
0·45% saline alone	41

* In each case the same volume of an equimolar solution of ammonium chloride was added to a tube of saline to serve as a control.

Plasma Potassium Level.—In blood samples stored with acid-citrate-dextrose the addition of RP.3300 resulted in a slightly increased loss of potassium from the cells into the plasma in most cases (Table VI). However, in samples stored with disodium citrate (without dextrose) the addition of RP.3300 in a final concentration of 1 in 10,000 and 1 in 20,000 produced no significant effects (Table VII).

Cell Swelling.—In blood stored with acid-citrate-dextrose + RP.3300 (final concentration 1 in 10,000) for one month the mean corpuscular haemoglobin concentration was consistently higher than in blood stored with acid-citrate-dextrose alone

TABLE VI

PLASMA POTASSIUM BEFORE AND AFTER STORAGE IN A.C.D. AT 4° C. FOR 31 DAYS WITH AND WITHOUT RP.3300 IN A FINAL DILUTION OF 1 IN 10,000

Blood Sample	Plasma Potassium (mg./100 ml.)			
	Before Storage		After One Month	
	With RP.3300	Without RP.3300	With RP.3300	Without RP.3300
H.C.	13*	13	112	91
P.L.M.	13+	14+	91	75
M.C.	13+	13+	109	105

* Values are the mean of estimates on two samples, except those marked +, which are based on single estimates.

TABLE VII

PLASMA POTASSIUM IN MG. PER 100 ML. AFTER 14 DAYS' STORAGE IN DISODIUM CITRATE AT 4° C. WITH AND WITHOUT RP.3300

Blood Sample	RP.3300 (1/10,000)	RP.3300 (1/20,000)	Without RP.3300
H.C.J.	101	102	97
P.L.M.	74	72	72
H.C.	82	68	71

(Table VIII). The slightly greater haemolysis of samples stored without RP.3300 was not taken into account in calculating mean corpuscular haemoglobin concentration and would in any case slightly increase the difference. It is concluded that red cells are slightly less swollen after storage with RP.3300.

Anti-sphering Effect on Red Cells.—Red cells sphered by repeated washing in normal saline buffered to *p*H 7.4 were instantaneously reconverted to biconcave discs when added as a weak suspension to buffered saline containing RP.3300 in concentrations of 1 in 10,000 to 1 in 80,000. This effect could then be reversed by

TABLE VIII

MEAN CORPUSCULAR HAEMOGLOBIN CONCENTRATION AFTER STORAGE FOR 31 DAYS IN A.C.D. AT 4° C. WITH AND WITHOUT RP.3300 IN A FINAL DILUTION OF 1 IN 10,000

Blood Sample		Mean Corpuscular Haemoglobin Concentration (%)	
		With RP.3300	Without RP.3300
H.C.	1.	27·8	25·4
	2.	27·2	25·6
	3.	28·2	26·2
M.C.		28·0	24·6
P.L.M.		26·9	24·4

washing the cells once and resuspending them in buffered saline. Since RP.3300 lowers the *p*H of unbuffered solutions, it was at first thought that the antisphering might merely be due to acidification as observed using dilute hydrochloric acid, or other soluble hydrochlorides, for example, adrenaline hydrochloride. However, in solutions buffered in the *p*H range 6.4–7.4, dilute HCl and adrenaline hydrochloride have no antisphering activity, whereas the activity of RP.3300 is apparently unchanged.

Effect on Red Cell Antigens.—Since RP.3300 seemed to exert its effects primarily on the surface of the red cell, it was decided to test its effect on agglutinability. Suspensions, 1 in 50, of red cells of group A, type M, Rh positive, were mixed with anti-M, anti-Rh, and anti-A sera, and the number of free cells was counted, after following the normal technique of differential agglutination. Red cells from the same donor, suspended in saline containing a final concentration of 1 in 40,000 RP.3300 and treated similarly, gave equally low counts. It was concluded that RP.3300 did not significantly affect the agglutination of human red cells.

Haemolytic Effects of RP.3300.—RP.3300 is haemolytic in concentrations exceeding 1 in 10,000, and the effect is increased by separating the red cells from their own plasma or by repeatedly washing them with normal saline. In this respect it resembles many other known lytic agents (saponin, bile salts, lecithin, etc.), and a time dilution curve (Ponder, 1948) plotted for 50% haemolysis was found to be essentially similar to those obtained for many other haemolysins. Similarly, the effects of temperature upon the haemolytic effects of RP.3300 were measured, and were essentially comparable to the temperature-haemolysis relationships of a number of other lytic agents.

Effect on Saponin Haemolysis.—It is well known that certain substances inhibit the haemolysis by saponin of washed red cells, while other substances act as accelerators of saponin haemolysis. Many of the " saponin accelerators " are themselves lytic, and, like RP.3300, many of them are surface-tension-reducing agents. RP.3300 in a dilution of 1 in 2,000 was found to be only slightly less lytic than saponin in a 1 in 100 dilution (50% haemolysis occurring in approximately 15 minutes in saline buffered to *p*H 7.4 and kept at 20° C.). However, when washed red cells were exposed to a solution containing both RP.3300 and saponin, at final concentrations of 1 in 2,000 and 1 in 100 respectively, total haemolysis occurred in less than 60 seconds. Lessening degrees of the accelerating effect of RP.3300 on saponin haemolysis could be demonstrated in dilutions up to 1 in 500,000.

Discussion

The results of the transfusion experiments show clearly that RP.3300 is not a red cell preservative in the sense of prolonging viability during storage. Indeed, the results suggest that the compound reduces viability.

It is necessary to reconcile this finding with the apparently beneficial effects of RP.3300 *in vitro*. The most striking of these effects is the retardation of spontaneous haemolysis. This can hardly be due to the diminished swelling of red cells stored in the presence of RP.3300, for this effect is a very slight one and, in any case, it has been shown previously that the spontaneous haemolysis of red cells during storage begins before the red cells have reached their critical volume (Mollison and

Young, 1942). It is possible that the spontaneous haemolysis of red cells during storage depends upon some enzyme activity with which RP.3300 interferes.

The present findings did not demonstrate any effect of RP.3300 on the rate of potassium loss from red cells stored in an acid-citrate solution. Although this result appears to contradict the findings of Halpern, Dreyfus, and Bourdon (1950), it should be noted that these workers used a different acid-citrate solution.

In a preservative solution containing dextrose it appears that RP.3300 increases the rate of loss of potassium from the red cells and this effect may be related to the diminished swelling of red cells stored in the presence of dextrose and RP.3300.

The immediate effects produced by suitable dilutions of RP.3300, i.e. antisphering and an increase in the resistance of red cells to hypotonic haemolysis, are presumably related to the " surface-activity " of the compound. Ponder (1948) notes that a number of haemolytic compounds, when used in sublytic doses, increase resistance to hypotonic haemolysis. Furthermore he points out that there are a number of substances which act as antisphering agents but that only plasma and plasma components can effect these changes without going on to produce haemolysis. It seems reasonable therefore to conclude that RP.3300 is a haemolytic substance which in sub-haemolytic concentrations produces a number of superficially favourable effects on stored blood but which exerts no fundamentally beneficial effects upon red cell preservation.

Summary

The addition of the phenothiazine derivative RP.3300 to blood does not increase its storage life, as judged by tests *in vivo*. Indeed, its effects appear to be mildly deleterious.

The apparently favourable effects produced by this compound *in vitro* resemble those of certain haemolytic substances, used in sublytic doses.

We should like to thank Boots Pure Drug Co. Ltd. for drawing our attention to the work of Dr. B. N. Halpern on RP.3300, and to Dr. Halpern himself for a generous supply of the compound and for letting us have details of an unpublished method for its estimation. We are greatly indebted to the director and staff of the North London Blood Supply Depot for allowing us to select suitable donors and for the collection of the blood. Miss A. Rogers, of the Department of Biochemistry in the Postgraduate Medical School of London, kindly performed the potassium estimations.

REFERENCES

Bing, F. C., and Baker, R. W. (1931). *J. biol. Chem.*, **92**, 589.
Dacie, J. V., and Mollison, P. L. (1943). *Lancet*, **1**, 550.
Domingo, W. R., and Klyne, W. (1949). *Biochem. J.*, **45**, 400.
Ebert, R. V., and Emerson, C. P. (1946). *J. clin. Invest.*, **25**, 627.
Halpern, B. N. (1947). *J. Allergy*, **18**, 263.
—— (1950). *Acta allerg. Kbh.*, **3**, suppl. 1, p. 164.
—— (1951). Personal communication.
—— and Bessis, M. (1950). *C.R. Soc. Biol.*, Paris, **144**, 759.
—— Dreyfus, B., and Bourdon, G. (1950). *Pr. méd.*, **58**, 1151.
—— and Hamburger, J. (1948). *Canad. med. Ass. J.*, **59**, 322.
—— and Reuse, J. (1949). *C.R. Acad. Sci.. Paris*, **228**, 514.
Loutit, J. F., and Mollison, P. L. (1943). *Brit. med. J.*, **2**, 744.
Mollison, P. L., and Young, I. M. (1942). *Quart. J. exp. Physiol.*, **31**, 359.
Parpart, A. K., Lorenz, P. B., Parpart, E. R., Gregg, J. R., and Chase, A. M. (1947). *J. clin. Invest.*, **26**, 636.
Ponder, E. H. (1948). *Hemolysis and Related Phenomena.* London.
Wurmser, R. (1946). *Rev. Hémat.*, **1**, 112.

J. clin. Path. (1952), **5**, 102.

A COMPARATIVE STUDY OF DIRECT MATCHING TECHNIQUES IN BLOOD TRANSFUSION

BY

O. G. DODGE

From the Blood Transfusion Centre, Sheffield

(RECEIVED FOR PUBLICATION NOVEMBER 10, 1951)

The discovery of several new blood-group systems within recent years and the development of new techniques for the detection of antibodies have shown that the time-honoured methods for the direct matching of blood preparatory to transfusion are inadequate and may be dangerously fallacious.

Tests in an albumin medium and the use of the indirect Coombs test are the most reliable methods so far developed for detecting incompatibility, and where direct matching which is not urgent is performed in hospital laboratories these procedures should, ideally, always be carried out. (It is, however, realized that this is not yet the case.) Ultimately, perhaps, all direct matching of blood will be carried out by pathologists, blood transfusion officers, and trained technicians working in laboratories equipped for the purpose. At present, however, a great deal of urgent direct matching, especially at night, falls to the lot of harassed and relatively inexperienced junior doctors who have neither the time nor the inclination to pursue the niceties of serological technique. Tube tests in albumin cannot satisfactorily be carried out in less than one and a half hours ; the indirect Coombs test requires practice and careful controlling to give valid results, and is, essentially, a laboratory test. Neither of these tests, however reliable their results, is therefore wholly satisfactory as a method for use in emergencies or for those who are not experienced in their use.

The purpose of this paper is to attempt (1) to show the fallacy of compatibility tests in a saline medium ; (2) to demonstrate the reliability of the tube technique of direct matching using an albumin medium ; and (3) to explore certain other methods of direct matching, in an endeavour to find a method which will combine speed and simplicity with the greatest possible degree of reliability.

Scope of the Investigation

It was decided to test a number of sera against cells of known ABO group and Rhesus type. The nature of the sera was not known to the investigator ; some contained anti-D in complete or incomplete forms (of varying titre) and some contained no immune antibodies. As some of the sera to be used contained anti-A or anti-B, cells of group O were used throughout. Errors in ABO grouping must now be very rare and the methods to be described would effectively reveal ABO incompatibility. In order to reproduce as closely as possible the conditions obtaining in hospitals, the blood samples were collected aseptically from bottles taken from the blood-bank of the Regional Transfusion Centre. The blood used was comparable to that issued

to hospitals, having been withdrawn from the donors from three to 20 days before testing.

In this investigation 72 sera, representing " patients' " sera, were each tested for compatibility with four different cell samples: three were O Rh positive and one was O Rh negative. A number of the sera were inert; the others were known to contain anti-D. Strongly reacting sera were not chosen, as it was hoped to find a method which would detect anti-D in the low concentrations usually found in patients. The anti-D sera had all been obtained from Rh negative women immunized by pregnancy, and their specificity and titres had all been confirmed by the M.R.C. Blood Group Reference Laboratory, Lister Institute. The sera were selected by an independent worker from the Sheffield serum panel, and the identity of each serum was not known to the investigator until the experiments were completed.

Altogether, eight different compatibility tests were used, although not every serum was tested against four cells by every method. Of the 72 sera tested, 11 proved to be inert and 61 to contain anti-D. Of these, the Blood Group Reference Laboratory had confirmed that 49 contained incomplete, three complete, and nine a mixture of complete and incomplete anti-D. The inert sera and Rh negative cells acted as controls.

Methods Used

Tile Method.—This was included mainly for historical reasons. To one volume of " patient's " serum on an opal glass tile was added one volume of an approximately 5% suspension of red cells in isotonic saline. The tile was rocked gently and then left to stand at room temperature. Readings were made after 20 minutes.

Tube Method with Saline.—To one volume of " patient's " serum in a precipitin tube was added one volume of a 2% saline suspension of red cells. The tubes were incubated at 37° C. for one and a half hours; the result was read microscopically by removing some of the sedimented cells with a pipette and spreading them gently on to a slide. Before the use of protein media was introduced this was the best available method for detecting immune antibodies.

Tube Method with Saline Followed by Centrifuging.—Serum and cells were mixed as above and incubated for 10 minutes in a 37° C. incubator. The tubes were then centrifuged for five minutes at approximately 2,000 r.p.m. The sedimented cells were gently agitated by passing two or three air bubbles through them with a Pasteur pipette, and removed and examined as before. Using this technique and reading the results macroscopically, Poole and Williams (1951) found it a reliable method of Rhesus typing—i.e., when unknown cells were being tested with a saline-agglutinating anti-D—but did not refer to its reliability in direct matching.

Tube Method with Albumin.—Serum and a saline cell suspension were mixed and incubated at 37° C. for one and a half hours as in the tube method with saline. The supernatant saline was then removed and replaced by an equal volume of 20% bovine albumin. The tubes were replaced in the incubator for a further 30 minutes. The results were then read microscopically. This technique has been found to give clear-cut and reliable results in Rh-typing and is economical in its use of albumin.

Diamond Open-slide Method.—One drop of serum was placed on the slide on top of a Diamond box and five or six drops of whole blood were added. The slide was rocked and readings were made after five minutes. Doubtful reactions, probably due to rouleaux, could usually be read more easily if a drop of saline was added.

The Diamond test is a useful quick method of Rh typing when selected, potent antisera are used; Diamond and Abelson (1945) detected anti-Rh agglutinins in nine Rh negative persons (known to have been immunized by transfusion of Rh positive blood) by this method when the saline tube method gave negative results. It is known, however, that the age of the red cells used greatly affects the sensitivity of this test, and, as Malone (1949) points out, it will fail more often in direct compatibility tests, where one is dealing not only with antibodies of unknown speed of reaction and potency but with stored cells of unknown sensitivity.

Godwin and McCall's Modified Diamond Method.—This method (Godwin and McCall 1949), and the one next described, differ from the Diamond method in that a longer time is allowed for the reaction, drying of the mixture is prevented, and the red cells are suspended in albumin instead of in their own serum.

A 50% suspension of the prospective donor cells was made in 20% bovine albumin. Equal volumes of the "patient's" serum and the cell-suspension (two drops of each) were mixed on a slide which was incubated, in a moist chamber, at 37° C. for 15 minutes. (A petri dish containing a pledget of damp cotton-wool, or some similar device, will serve.) The slide was then removed and the results read by placing over a light and rotating the slide. If agglutination has taken place, the disc of sedimented cells has a serrated edge and on rotating the slide the disc breaks up into clumps, usually easily visible to the naked eye.

"Australian" Modified Diamond Method.—This is a modification of a method for detecting saline agglutinins described in Australia by Krieger and Weiden (1948).

A 5–10% suspension of "donor" cells in 20% bovine albumin was prepared. To one volume of the "patient's" serum on a slide one volume of the cell suspension was added and the whole incubated in a moist chamber at 37° C. for 30 minutes. Agglutinated cells formed an irregularly-edged disc which, when the slide was rotated, broke up into clumps which were in most cases easily visible to the naked eye.

Indirect Coombs Method.—In the hands of trained workers this is undoubtedly the most sensitive method for detecting antibodies. Many antibodies can in fact only be detected by this technique (e.g. anti-Fy^a and some anti-K). Owing to the scarcity of anti-human globulin, each serum was tested by this method against only one Group O Rh positive cell sample. The anti-human globulin was controlled on each day of the investigation, using cells sensitized with a known incomplete anti-D, and unsensitized cells, as positive and negative controls respectively.

Results

The results are summarized in Table I. A positive result was taken to indicate incompatibility, a negative to indicate compatibility. By comparing the results obtained with the actual antibody-content of the sera used, as was done at the end of the experiment, it was possible to estimate the relative sensitivity of the methods used in detecting the presence of immune antibody. The compatible and incompatible results could then be divided into "true" and "false." The most important figure was that of the "false compatibles," i.e., those where no agglutination took place when known Rh positive cells were mixed with a "patient's" serum containing anti-D.

In the saline and Diamond tests a number of doubtful reactions were seen, consisting of unevenly distributed clumps of two to three cells in the former, and a faintly granular appearance just visible to the naked eye in the latter. The figures in Table I have been obtained by recording all these doubtful results as positive (incom-

TABLE I

RESULTS OF DIRECT COMPATIBILITY TESTS CARRIED OUT BY SEVEN DIFFERENT METHODS

Method Used	Total Tests	Total Incompatible (Positive)		Total Compatible (Negative)		Incompatibility				Compatibility			
						"True"		"False"		"True"		"False"	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Tile technique	256	4	1·6	252	98·4	4	1·6	0	0·0	94	36·6	158	61·8
Saline tube	272	140	51·5	132	48·5	135	49·7	5	1·8	90	33·1	42	15·4
Saline tube with centrifuging	274	101	36·9	173	63·1	101	36·9	0	0·0	102	37·2	71	25·9
Albumin tube	274	173	63·1	101	36·9	172	62·8	1	0·3	101	36·9	0	0·0
Original Diamond	270	72	26·7	198	73·3	70	25·9	2	0·8	97	35·9	101	37·4
Godwin and McCall (modified Diamond)	274	166	60·6	108	39·4	162	59·1	4	1·5	98	35·8	10	3·6
"Australian" modified Diamond	273	175	64·1	98	35·9	163	59·7	12	4·4	88	32·1	10	3·8

patible). In this way the number of "false compatibles" has been reduced to a minimum, and the figures for each method made as far as possible comparable.

Albumin Tube Method.—This gave the most accurate results. No "false negatives" were obtained with this method. In no case did the cells and serum appear to be compatible when they were not so. There was one "false positive": an Rh positive cell gave a W reaction with an inert AB serum. This result could not be repeated and is presumed to be due to technical error. Unagglutinated cells tend to look "sticky" in albumin, but this appearance is quite distinct from the very large clumps easily seen in the positives and there was never any doubt whether a given tube showed a positive or negative result.

Saline Tube Method.—It will be seen from Table I that this method is much less sensitive than the albumin technique because it fails to detect incomplete anti-D in "patient's" sera.

Saline Tube Method with Centrifuging.—This method is rapid, but may possibly give rise to two errors in interpreting the results, due to (1) mistaking groups of sedimented cells for agglutinates; (2) breaking up agglutinates when resuspending the sedimented cells.

This method has shown itself considerably less sensitive than the standard saline tube technique in this investigation, and, like it, fails to detect albumin-agglutinating anti-D.

Tile Technique.—This is included for the sake of completeness. In 256 tests, only four positive results were obtained. Three of these were with an anti-D serum having a titre in saline of 64, the most powerful serum used in the investigation.

Indirect Coombs Test.—This test is capable of detecting antibodies that cannot be revealed either by saline or albumin techniques. In the present series, however, all the antibodies present were detected by the albumin tube technique. Nearly all the sera were tested by the indirect Coombs test, one sample only of O Rh positive cells being sensitized by each serum. The results showed that of 56 anti-D sera 55 gave a positive indirect Coombs test with one negative result, whilst 10 inert sera gave 10 negatives.

The one negative Coombs test with known anti-D serum could not be repeated and was presumably due to technical error.

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis.

Interpretation of Results

It must be emphasized that the figures obtained refer only to the *relative* sensitivity of the various methods in detecting a particular type of immune antibody (anti-D). As far as safety in transfusion is concerned, the most important figures are the "false negatives," those tests which gave a result indicating compatibility between patient's serum and "donor's" cells, when, in fact, the serum contained an antibody capable of agglutinating those cells. The percentages of such results for the various methods are given in the right-hand column of Table I. These results show the fallaciousness of compatibility tests in a saline medium, and the reliability of compatibility tests in an albumin medium.

The albumin-tube technique emerges as the most reliable method of detecting weak, incomplete anti-D. It should always be carried out when a direct matching test is requested. Its only disadvantage is that it takes about two hours. The search for a method that will give reliable results in a shorter time has shown that the two modifications of the original Diamond test are superior to the other rapid methods used. Each method detected over 90% of the antibodies employed. Where 100% reliability has to be sacrificed to considerations of speed, and when the help of laboratory personnel is not available, one of these methods provides the greatest safety, with the Godwin-McCall giving quicker readings than the "Australian" method. An indirect Coombs test should be performed whenever possible in order to detect those antibodies which can only be demonstrated by that method.

"False Negative" Results in the Modified Diamond Tests.—In view of the high accuracy of these tests an assessment was made of avoidable factors producing occasional "false negatives." Possible causes are (1) low-titre sera, (2) storage-period of cells, (3) weakly-reacting cells, and (4) errors of technique or interpretation. It appeared that the use of low-titre sera and the length of time the cells had been stored were not related to these "false" results. Three of the cell-samples used gave consistently weaker reactions than other cells by all techniques, and these weakly reacting cells gave "false negatives" in the modified Diamond tests. Finally, three "false negatives" with the "Australian" method were recorded early in the investigation, when little experience of this technique had been gained, and these results may have been due to error of interpretation.

Summary

The relative sensitivity of eight types of direct compatibility test was estimated by comparing their ability to detect weak immune antibodies (anti-D). The great reliability of tube tests using albumin was demonstrated, as was the insensitivity of similar tests in a saline medium. Of the rapid methods of direct matching, the most reliable were found to be two modifications of the Diamond test. The occasional "false negative" results given by these methods did not show any definite correlation with the age of the cells, or the titre of the sera used. When direct matching which is not urgent is done in hospital laboratories, a tube-test using albumin (and perhaps the indirect Coombs test) should always be carried out. When urgent direct matching has to be carried out and laboratory facilities are not available, the modified Diamond methods described appear to give the most trustworthy results.

I wish to express my thanks to Dr. R. H. Malone, director of the Blood Transfusion Centre, Sheffield, for permission to publish this paper ; and to him and Mr. I. Dunsford, chief technician, for their valuable encouragement, criticism, and advice.

REFERENCES

Diamond, L. K., and Abelson, N. M. (1945). *J. Lab. clin. Med.*, **30**, 204.
Godwin, B., and McCall, A. J. (1949). *Brit. med. J.*, **1**, 870.
Krieger, V. I., and Weiden, S. (1948). *Amer. J. clin. Path.*, **18**, 572.
Malone, R. H. (1949). *Brit. med. J.*, **1**, 1003.
Poole, J. C. F., and Williams, G. C. J. (1951). *Journal of Clinical Pathology*, **4**, 55.

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis

J. clin. Path. (1952), **5**, 108.

TECHNICAL METHODS

A NEW TYPE OF BLOOD FILM FOR DIFFERENTIAL COUNTS

BY

MICHAEL F. A. WOODRUFF

From the Department of Surgery, University of Aberdeen

(RECEIVED FOR PUBLICATION MAY 23, 1951)

Determination of the lymphocyte count is becoming of increasing importance and would undoubtedly be undertaken more frequently, for example in patients under treatment with cortisone or A.C.T.H., if the standard procedure were not so tedious.

Some help is afforded by the use of machine-made blood films as described by Marks, Bailey, and Gunz (1950) because the number of cells which must be counted to attain a given degree of accuracy is smaller than in the case of hand-made films, but the labour involved is still considerable. Enumeration of lymphocytes directly in the haemocytometer has been suggested as an alternative but is, in the writer's experience, difficult and not altogether reliable.

A new type of thick blood " film " has therefore been designed which enables an accurate differential count to be performed rapidly and has the further advantage that a reliable estimate of the standard error can be made.

Routine Procedure

An ordinary draughtsman's ruling pen is prepared by coating the blades with a silicone preparation (Dri-film 9987, American General Electrical Co.) so that their surface becomes non-wettable. The pen is then loaded with a drop of blood and a " film " consisting of one or more lines, each approximately 0.25 mm. wide, is ruled on an ordinary glass slide as shown in Fig. 1. To obtain a uniform and at the same time thick " film " the pen is drawn backwards and forwards four or five times over each line. The slide is left for some hours to dry, or alternatively is dried more rapidly in a warm but not hot oven. It is then stained with Field's stain by the method normally used for the staining of thick blood films for malaria diagnosis. The red cells are all lysed, and, provided care is taken to avoid overstaining, the distinction between polymorphonuclear and mononuclear cells is easily made. The further subdivision of the latter into lymphocytes and monocytes is more difficult, but this, fortunately, is seldom important in the type of work for which the film was designed.

Counting is best done with an oil-immersion objective (1/12 in. or 1/8 in. if available). A start is made by counting all the cells in a narrow strip of width equal to the diameter of the field and running right across the line at some convenient site. The slide is then moved to right or left a distance equal to the diameter of the field and another strip counted. By repeating this procedure all the cells in any desired segment of the line are included.

FIG. 1.—The slide is held in an ordinary slide tray. The photograph has been taken from an unusual angle to show details of the pen, and gives the impression that the latter is held obliquely. In reality, however, it is held almost normally to the plane of the slide.

Owing to the thickness of the film the leucocytes are closely packed, there being in a typical case an average of seven cells per field. A given number of cells may therefore be counted in a much shorter time than would be required with a conventional film. It took the writer, for example, 17 minutes to count 1,000 cells on a line film and an hour to do a battlement count of 1,000 cells on a conventional film from the same individual.

Statistical Investigation of the Cell Distribution

Blood from the finger of a healthy young adult was transferred with a siliconed pipette to the pen and successive lines were then ruled until all the blood had been used ; 7 ml. gave in all eight lines each 6 cm. long.

After the films had been dried and stained every leucocyte in the whole of the first line and in a randomly chosen segment 1 cm. long from each of the other lines was counted, each cell being classified as either a polymorphonuclear or mononuclear cell. A record was made of the number of cells of each type in successive strips of width equal to the field diameter (approximately 0.15 mm.), and from this the number in each successive millimetre or centimetre was obtained. In addition the number of mononuclear cells in each successive 100 total cells was noted. For comparison a total leucocyte count was performed at the same time in a haemocytometer and a conventional film was also made. A differential count was performed on the latter using the standard battlement technique described in a previous paper (Woodruff and Forman, 1950). One thousand cells were counted, the proportion of mononuclear cells in each successive 100 total cells being recorded as before.

In the haemocytometer 327 leucocytes were counted giving a total leucocyte count of 7,670 cells per c.mm. with a standard error of $\pm$ 440 cells per c.mm., assuming that shaking of the pipette was sufficient to give the theoretical Poisson distribution. The estimated total count by the line film was 50,705 cells per 7 c.mm. blood, i.e. 7,240 cells per c.mm. There is clearly no significant difference between the two results, from which it may be concluded that all the cells in the line film can be accounted for.

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis.

The 10 successive hundred cells in the battlement count gave a mean value of 39.50% mononuclear cells, with a standard deviation of ± 1.5%. The distribution conformed well to a binomial distribution ($\chi^2_{[9]} = 8.4$, $P \simeq 0.5$). This is of interest since Marks *et al.* (1950) found a binomial distribution in a series of counts of 100 cells from machine-made but not from hand-made films. It may be pointed out, however, that the hand-made film used in the present investigation was made and counted by a carefully standardized procedure.

The mononuclear cell count for the 69 hundreds in the first line of the linear film conformed well to a binomial distribution ($\chi^2_{[68]} = 57.8$, $P \simeq 0.8$). The 6 cm. of this line were further tested for homogeneity and gave a satisfactory result ($\chi^2_{[5]} = 4.3$, $P \simeq 0.5$). The 7 cm. from the other seven lines were also tested for homogeneity and proved satisfactory ($\chi^2_{[6]} = 4.5$, $P > 0.6$) and so did the whole 13 cm. tested together ($\chi^2_{[12]} = 9.6$, $P > 0.6$). Accordingly it was possible to give a mean value for all the readings on the linear film, and this came to 40.82% ± 0.41% mononuclear cells.

The difference between the battlement and linear estimate is clearly not significant (1.32% ± 1.61% mononuclear cells).

Summary and Conclusions

This type of film should prove to be of great value when a rapid but accurate estimate is required of the proportion of polymorphonuclear cells on the one hand and of lymphocytes and monocytes together on the other hand. One thousand cells may be counted in about 15 minutes, and since the distribution corresponds closely to the binomial the standard error may be calculated from the following formula:

$$\text{S.E. (expressed as \% of polymorphs or mononuclears)} = \sqrt{\frac{P\ (100-P)}{n}}$$

where P = % polymorphs and n = total number of cells counted. Thus, for example, if P = 50%, the S.E. for a count of 1,000 cells is ± 1.58%. For the same proportion of polymorphs if only 100 cells are counted (taking no more than two minutes) the S.E. is ± 5%.

In addition to saving time the line type of film saves space. This is particularly the case when several successive counts are required from one individual, as six to eight lines, each representing one count, may be placed on a single slide.

The application of this type of film to the diagnosis of malaria is obvious.

One possible refinement has been considered, namely, the use of a line film equal in width to the diameter of the microscope field, so that the slide need be moved in one direction only. It has been found possible to achieve this for a 1/8 in. but not for a 1/12 in. objective. If, therefore, an oil-immersion 1/8 in. objective is available this modification is worth trying.

The writer is grateful to Mr. F. H. C. Marriott for advice regarding the statistical procedure and for checking the computations.

References

Marks, J., Bailey, N. T. J., and Gunz, F. W. (1950). *Journal of Clinical Pathology*, **3**, 168.
Woodruff, M. F. A., and Forman, B. (1950). *Brit. J. exp. Path.*, **31**, 306.

J. clin. Path. (1952), **5**, 111.

A NEW COUNTING CHAMBER FOR THE EOSINOPHIL COUNT

BY

T. MANNERS

From the Department of Pathology, Royal Infirmary, Sunderland

(RECEIVED FOR PUBLICATION JULY 20, 1951)

During the past four years numerous articles have been published in which eosinophil counts have been used in the control of therapy by A.C.T.H. In most of these the counts have been performed by using four Fuchs-Rosenthal counting chambers for each count. The author also used these counting chambers for the experimental work on a new diluent for eosinophil counts (Manners, 1951), but found the method cumbersome. Much time was wasted in filling and cleaning four counting chambers when only one should have been necessary. While counting, it was necessary to move to the next counting chamber three times and to refocus if the counting chambers were of different thickness. These disadvantages are obviated by the use of a new counting chamber* designed by the author.

The ruling in the new chamber is similar to that of the Fuchs-Rosenthal in that it is composed of large squares 1 mm. × 1 mm., each divided into 16 smaller squares. The ruled area is 4 mm. × 12.5 mm., and the depth of the chamber is 0.2 mm. The whole ruled area is counted and the volume enclosed by it is 10 c.mm. As the dilution of blood is 1 : 10, no calculation is required to find the number of eosinophils per c.mm. When using four Fuchs-Rosenthal chambers the number counted has to be divided by 1.28 to obtain the number of eosinophils per c.mm.

Plum (1936) showed that the accuracy attainable in chamber counting depends for the most part on the number of cells per sample counted. There is a great variation due to chance distribution in the number of cells from sample to sample of a given blood, and its standard deviation (S.D.) is equal to the square root of the mean number of cells per sample. As the volume of fluid contained in the new counting chamber is slightly smaller than the total volume contained in four Fuchs-Rosenthal counting chambers, the accuracy of the count is slightly less. The theoretical accuracies of the two counting techniques can be compared by taking an example.

With a blood containing 100 eosinophils per c.mm., the counts obtained in the new counting chamber would average 100 cells. The S.D. of their variation due to chance would be $\sqrt{100}=10$, which is 10% of the mean. Using four Fuchs-Rosenthal chambers, which have a total volume of 12.8 c.mm., the count would average 128 cells. The S.D. in this case would be $\sqrt{128}=11.3$, which is 8.8% of the mean. With 100 eosinophils per c.mm. the comparison is thus between an S.D. of 10% and 8.8%.

The difference will be less at higher levels and greater at lower levels. The difference is small and the accuracy attainable in the new chamber is sufficient for routine counting of eosinophils.

REFERENCES

Manners, T. (1951). *Brit. med. J.*, **1**, 1429.

Plum, P. (1936). *Acta med. scand.*, **90**, 342.

* Made by Messrs. Hawksley and Sons, Ltd., 17, New Cavendish Street, London, W.1.

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis.

J. clin. Path. (1952), **5**, 112.

COMBINED STERNAL BIOPSY AND ASPIRATION NEEDLE

BY

B. A. THOMPSON

From the Group 9 Pathological Laboratory, Peace Memorial Hospital, Watford

(RECEIVED FOR PUBLICATION OCTOBER 18, 1951)

In the August issue of this journal Bernstock and Sterndale (1951) described a combined sternal biopsy and aspiration needle, using as a basis a Sahlah type marrow needle. Before publication of this device we had made a similar type of needle, using as a basis a Waterfield (1946) sternal needle. From experience we have found that the Waterfield pattern gives greater control while performing the puncture; the screw thread type guard is more easily adjusted and gives a greater degree of safety over the slip type guard of the Sahlah pattern.

As shown in the photographs, the principle of the combined needle is the same as that of Bernstock and Sterndale, namely an outer needle of the Waterfield pattern with a bore of 14 British wire gauge, and an inner needle consisting of a trephine with a bore of 17 British wire gauge. The upper ends of both the outer and inner needles have a Record syringe fitting.

We should like to thank Messrs. S. J. Owen, of 35 Northampton Square, London, E.C.1, for making both inner and outer needles; and Dr. E. Elkan for the photographs.

REFERENCES

Bernstock, L., and Sterndale, H. (1951). *Journal of Clinical Pathology*, **4**, 378.
Waterfield, R. L. (1946). *Lancet*, **1**, 576.

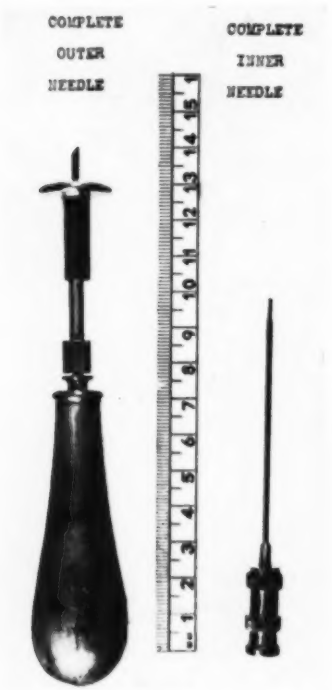


FIG. 1

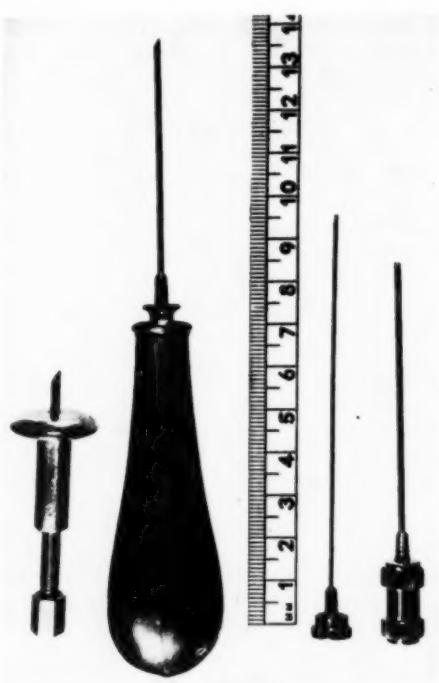


FIG. 2

FIG. 1—Outer needle and trephine with stylet *in situ*.

FIG. 2—Both needles dismounted showing component parts.

J. clin. Path. (1952), **5**, 113.

REVIEWS

Recent Advances in Clinical Pathology. By various authors (general editor S. C. Dyke). 1951. London: J. and A. Churchill, Ltd. 2nd edit., with 37 plates and 36 text figures. Pp. 575. Price 40s.

This edition, which has appeared only four years after the original publication, is really a new volume of the "Recent Advances" series and, in fact, it is clearly stated that this issue does not supersede, but is definitely supplementary to, the first edition. Three-quarters of the subjects are new and, while the total number has been reduced by 10, the text is increased by almost 100 pages. These facts indicate the rapid and extensive developments in clinical pathology and it is therefore unfortunate that the publication of this edition was unduly delayed. There are very few references to work published in 1951 and it would appear that most of the manuscripts were prepared in 1950 or possibly earlier.

The subjects have again been divided into four main sections of approximately equal size: bacteriology, biochemistry, haematology, and histology, with a short additional chapter on laboratory design and equipment. Three of the sectional editors remain, namely, R. Cruickshank (bacteriology), E. N. Allott (biochemistry), and A. H. T. Robb-Smith (histology), but R. G. Macfarlane (haematology) replaces B. L. Della Vida, who has returned to Rome. The new chapter, written by Dr. J. E. McCartney, should prove invaluable to the many pathologists who are hoping to extend their departments in the near future, as it includes much useful information and advice on the construction and detailed fittings of laboratories. It must, however, be stated that the recommended accommodation for a large group laboratory seems grossly inadequate, particularly in view of many new, and sometimes complicated, tests described in the previous sections of this book.

The technical subjects have been well selected. They cover a wide range and, as they have been written by accepted experts in their respective fields, the general standard is high. There is a tendency to excessive compression, but most contributors have managed to include in their comprehensive surveys a useful practical summary of the appropriate techniques. In the case of bacteriology, the inclusion of the addresses of the many reference laboratories now available for special tests is welcome. Some points, which may be subjected to minor criticism, are the statement on p. 43 that the estimation of chloride (C.S.F.) is useless in the diagnosis of tuberculous meningitis; the description of several long-established techniques in the chapter on blood analysis; the absence of comment on the Chown test and halometry in the discussions of Rh tests and M.C.D. respectively.

This edition can be thoroughly recommended to all interested in the various aspects of clinical pathology, and it is a pleasure to congratulate the general editor and his colleagues on their excellent and well-balanced production. A further edition will doubtless be demanded and it is hoped that attention will then be given to the format of the book, which is now becoming inconvenient to handle.

R. W. FAIRBROTHER.

Muir's Text-book of Pathology. Sixth edition revised by D. F. Cappell. 1951. London: Edward Arnold & Co. Illustrated. Pp. xx+1,090. Price 50s. net.

A textbook on pathology would seem to be, by tradition, associated with the Chair of this subject at Glasgow. As long

• Megaloblastic haemopoiesis. † Normoblastic haemopoiesis

ago as 1883 Joseph Coats, whose supremacy as a pathologist in the west of Scotland—on the authority of Sir William Gairdner—" was unquestioned, and he had the countenance and support of the University long before he became a professor *de facto*," produced the first edition of his Manual. Coats was only appointed to the Chair five years before his death in 1899, but his work endured as a students' textbook for fully 10 years after 1903, when the fifth and posthumous edition was edited and revised by Louis Sutherland of St. Andrews. Coats was succeeded by Robert Muir, who entered into the Institute which had been called into being largely by the initiative of his predecessor, and in 1924 produced his own textbook which under his hand ran into five editions and seven reprintings, the last being in 1946. Muir's textbook bore the stamp of his wide experience and mature judgment. It was sparing of frills, economical of words and, perhaps, almost austere in confining a subject which has such wide ramifications and lies at the basis of medical thought and practice within the manageable limits of a quarto volume of 991 pages. Nevertheless, as a source of sound pathological teaching, based upon personal observations, this textbook has been constantly remarkable. Within a limited compass was packed an immense amount of information, and there can be few pathologists in this country, even amongst those of the widest experience, who in times of doubt have not had a look within its red covers as a wise precaution before committing themselves upon any disputable theme.

In the sixth edition which is now before us Professor Cappell continues in the Glasgow tradition both historically and one might almost say geographically, for he, too, like Sutherland, held a Chair at St. Andrews. The format and general style of the sixth edition are unchanged, but the book has been very extensively revised. Increase in size, by the addition of about a hundred pages, has been attained without noticeable increase in volume by the use of a thinner and more glossy paper, which improves the appearance of the page and sets off the illustrations to better advantage. These have been increased from 599 to 636 and are almost wholly photographs. Their quality is uneven and some of them, e.g., Nos. 36, 75, 497, and 628, are not very informative, whilst the student might well complain that Figs. 508 and 510 look to him to be very much the same. Fig. 78 is poor and too darkly printed. On the credit side, however, some of the newer ones, e.g., Figs. 38, 63, 287*a* and *b*, are excellent. It is a moot question whether photomicrographs are the best means of illustrating a book used by students beginning the subject of pathology. Their accuracy, of course, is their undeniable virtue, but they do require some experience before they can be properly interpreted. It can, of course, be argued that the student may as well begin as he means to go on, and that as in the future most of the histological illustrations he sees will be photomicrographs, so he may as well be broken in to them at once!

Professor Cappell has achieved a notable success in this revision, which cannot have been an easy task, if for the single reason alone that the incorporation of new knowledge in an already well balanced presentation of a subject is always difficult. This, however, has been scarfed in so skilfully that no joints are noticeable. Selye and the " adaptation syndrome " get the better half of a page (one wonders how much they will have in the tenth edition), Fisher's Rh convention, fibrocystic disease of the pancreas, lymphogranuloma inguinale and granuloma inguinale (human ingenuity could scarcely have devised a more confusing nomenclature), sarcoidosis (described on page 99 and illustrated on page 539), protruded discs, agenized flour—all are there. There is a pleasing simplicity in the treatment of the reticuloses. We note the attribution to Heggie of an earlier observation of the " Trueta " or " Oxford " renal shunt. Perhaps it is confusing that lipomatosis of the heart wall is also called fatty infiltration, more especially as it is also conceded that excess of fat in the

parenchymatous cells of myocardium, liver and kidney is an infiltration—a view which is doubtless correct. A new and welcome feature is a short bibliography of monographs.

" Muir," as it is long likely to be called, is in safe hands and continues to be, as it has always been, a good sound textbook of British pathological teaching. The new edition worthily unholds the high tradition of a great school of pathology and a famous nursery of pathologists.

J. H. DIBLE.

Blood Transfusion in Clinical Medicine. By P. L. Mollison. 1951. 59 illustrations. Pp. 456. Oxford: Blackwell Scientific Publications. Price 37s. 6d.

The arrival of this new book will be widely welcomed by workers in the field of clinical pathology and transfusion techniques. It sets out to cover the whole field of transfusion, including the clinical laboratory and experimental aspects. The first chapters are in the main theoretical, and consider at considerable length the present knowledge of what happens to the chemical and cellular components of blood on storing. Modern methods such as differential agglutination are compared with other estimations using radioactive materials, and their close agreement is shown, for example, on the survival time of red cells. The effect of storage on the survival time of red cells after transfusion is also considered, and ideal recipes for preserving solutions are described. Blood volume techniques are compared, and physiological and pathological variations briefly discussed. An excellent chapter on the life span of red cells shows the wide variations which are found when cells from patients suffering from different diseases are compared, and much of this chapter recapitulates the author's original work. The indications for transfusion in such conditions as haemorrhage, burns, anaemias, and leukaemias are also discussed, and particularly valuable will be the warning regarding transfusions in severe anaemias, and the safety measures which must be adopted to prevent overloading.

Then follow four chapters on the blood groups and blood grouping techniques. As we would expect from the author, these are most carefully and clearly computed, and the newer blood-group systems are adequately described. In the chapter on techniques, details of preparation of antisera and elution techniques are particularly well done and easily followed. The author has a practical approach to the vexed problem of compatibility tests, and divides the immediate and urgent techniques from those which can be done at leisure. This journal has published several differing papers on compatibility techniques, and readers will readily accept Mollison's conclusions that carefully controlled techniques which provide complete safeguards must be adopted to prevent transfusion reactions. The author recommends as the standard procedure a cross-matching technique, in which a 2% suspension of donor's cells in 20% albumin is used, and the results read after two hours' incubation. This procedure need only be modified in special cases where there have been previous transfusions, possibly with reactions, or any chance that the patient's blood contains immune iso-antibodies. In these cases in view of the possibility of zoning and of antibodies which do not agglutinate cells in albumin—e.g., Duffy—the ordinary test in albumin should be supplemented by the indirect Coombs test. Deviation from these rules should be allowed only in real emergency, and here the author prefers the modified Diamond slide test. In this issue an investigation by Dodge arrives at an identical conclusion (p. 102).

Transfusion reactions, including haemolysis, transmission of infection, reactions to pyrogens, allergic reactions, air embolism, and jaundice are well described in the following chapters, and the book ends with an excellent description of the normal blood pictures in newborn infants, haemo-

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis.

lytic disease of the newborn, and indications for transfusion in the newborn. The criteria for anaemia in the neonatal period and the indications for transfusion are amongst the best sections of the book, and the technique of transfusion in the newborn describes in detail the use of the umbilical vein, the scalp veins, and the saphenous vein.

The whole book shows a mastery of the subject, and for the first time collects into one volume the wider as well as the more detailed aspects of transfusion techniques. It is a book which should be read by the clinician, who will probably skip many of the mathematical data, but will gather an idea of the difficulties of the laboratory worker; and should certainly be read by every practising pathologist who has to control blood collection, storage, or transfusion. It is easy to prophesy that this will become a standard textbook in British hospitals.

One or two minor criticisms are mainly of faulty proof-reading, and in some parts the author is difficult to follow in the concentrated discussions. These do not detract from the overall ease of following the author's meaning, particularly in the technical sections.

A. GORDON SIGNY.

Observations on the General Effects of Injury in Man. (With Special Reference to Wound Shock.) By R. T. Grant and E. B. Reeve. 1951. Special Report Series No. 227. London: His Majesty's Stationery Office. Pp. xiii 313. Price 8s. 6d.

This special report is based on the investigations made during the last war on 230 cases of injury to the limbs and on 80 cases of injury to the abdomen. The patients were drawn from the home front and the Italian campaign. The investigations were confined to injury in two parts of the body, the limbs and the abdomen, and so the authors, with admirable discretion, have set out to examine in detail a limited problem. The report is also enhanced by the fact that the cases were studied from the time of admission to hospital until recovery seemed assured or until death. In this respect this report would seem to be unique.

Throughout these investigations special attention was paid to the study of the circulation and to four factors in particular, blood pressure, pulse rate, skin temperature of the extremities, and colour of the face, and the clinical condition was assessed by these simple bedside methods. The blood volume was measured on 71 cases of the Italian series, but was not used in the study of the casualties on the home front.

This report again emphasizes that the central feature in shock is the fall in blood volume. The amount of this fall can be reasonably accurately assessed by a study of blood pressure and of the size of the wound. A method is described of estimating the wound size in terms of the size of the hand and will be found to be one of great value in everyday work in the casualty ward.

The main cause of the fall in blood volume is haemorrhage, and so in treatment one must concentrate on intravenous transfusion of blood, plasma, or serum. These substances must be given in sufficient quantity, sufficiently early, and at a sufficiently rapid rate to restore blood volume at the earliest possible moment, and this appears to be the main practical lesson to be learnt from this report. Gone are the days when the patient was allowed to lie for several hours to recover sufficiently for operation to begin.

Due credit is given to the work of investigators in the first world war, especially to Keith, who was the first to show by measuring the blood volume (vital red method) that wound shock is considerably associated with a reduced blood volume and that the degree of reduction bears a definite relationship to the severity of the patient's clinical condition. Grant and

Reeve find no evidence of a toxic origin of shock and the nervous stimuli are only a secondary factor. Haemorrhage is the essential cause of the fall in blood volume, and where cases do not respond to transfusion then the blood volume has either not been restored at all or not restored quickly enough. This is true in the main, though it is admitted (Case B 60) that in some cases other factors operate. Having cleared the ground of much that is obsolete, the report introduces two other factors which play a part in the shock syndrome, fat embolism and disorder of mineral metabolism. The authors believe that fat embolism plays a part, how large they are not quite sure, in the clinical course of the shocked patient, though they are quite sure it is not a major factor in the production of shock. This is probably the most controversial part of the report. It may come as a surprise to those who are not conversant with the literature in this field that these workers found that perhaps 20% of those dying of abdominal wounds might have been saved and the clinical course of those who recovered made easier if salt and fluid had been given.

The final part of the report discusses the more technical aspect of the work on blood volume measurements and haematological and biochemical changes associated with injury. This section is wholly admirable. The methods of estimating blood volume using the dye T1824 are analysed and their application to the estimation of haemorrhage outlined ; the volume of transfusion required to restore blood volume is discussed in detail. It is refreshing to find that the simple haemoglobin estimation can be of value in the estimation of blood loss, and the field worker is once again indebted to these practical workers. Many workers, especially Moon, have stated that haemoconcentration is an essential part of shock. Grant and Reeve found that this is not true ; in fact haemodilution occurs in most cases. In the case of limb injuries after blood loss there is a fairly rapid dilution of the blood first with fluid poor in protein, but later and chiefly with fluid containing protein in nearly the same concentration as the original plasma. In abdominal injuries with penetrating intestinal wounds haemoconcentration may occur after the first 12 hours due to dehydration.

If any criticism can be made of this carefully documented report by two such famous investigators it would be that in some cases the necropsy reports are not very complete ; the histological findings especially could have been a little more detailed. For instance, it would have been of value to have known more of the histological examination of the brick red lungs of the most interesting case, I 5, as surely the possibility of blast injury arises. In fact one is struck throughout the whole report by the fact that little or no consideration has been given to the possibility of blast lesion. Again no attempt appears to have been made to examine the urine or sputum for fat droplets during life, and no mention is made of the examination of the fresh lung for fat emboli, a method found to be of great use by other workers.

It is an indication of the rapid way in which work in this field is progressing in that since the work was completed the lesson of speed in transfusion has been carried to the stage when intra-arterial transfusions are now given, the type of fluid which can be given has been increased by synthetic substances, and the study of sodium metabolism has been shown to be at times less important than derangement of the potassium ion. However, this is a report which must be read by all those interested in the treatment of the injured patients and will not only serve as a record of careful work but will, like all good research, no doubt act as a guide to all future workers in this field.

The bibliography is helpful and reasonably complete, though, with the emphasis laid on fat embolism throughout the work, one would have expected a little more consideration given to the work of others on this subject.

J. V. WILSON.

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis.

Syndromes Nouveaux de Pathologie Adipeuse. By William A. Blanc. 1951. Paris: Masson & Cie. With 84 text figures. Pp. 256. Price Fr. Fr. 1,200.

In the introduction to this book the author suggests that little attention has been paid to the problem of fat necrosis. The first chapter deals with the anatomy and physiology of adipose tissue and the second with experimental work on subcutaneous and intravenous injection of fats. Two chapters are then devoted to fat necrosis, both focal and diffuse, and the various disease syndromes which it produces. The fundamental local lesion progresses from necrosis and cyst formation to laminated hyalinization and perhaps calcification. Phagocytosis of the fat from the dead cell by histiocytes and its deposition at a distance, where it acts as a foreign body, is the mode of spread of the lesions. The author considers that humoral protein imbalance is a factor in the production of progressive liposclerosis. In the last chapter he discusses the interrelationship of all connective tissue diseases, and suggests that the pathological changes in liposclerosis are similar to those in amyloid disease and other disturbances of protein metabolism.

This is an exhaustive study of the pathology of fatty tissue, and much of the monograph is repetitive, particularly the chapters on fat necrosis. The photographs also tend to show the same repetitive quality. A long classification of fat necrosis is given, which the author considers incomplete, and descriptions of the lesions are repeated much too often. In some cases the description suggests that changes in the fatty tissue are merely part of a diffuse polyarteritis, and at one point the author admits the possibility of primary vascular disease followed by necrosis of the surrounding fat. A further criticism of this book is that, although the title is *Syndromes Nouveaux de Pathologie Adipeuse*, most of the syndromes were described many years ago, some even last century.

M. GILLESPIE.

Foetal and Neonatal Pathology. By J. Edgar Morison. 1951. London: Butterworth & Co., Ltd. 1st edit., with 59 illustrations. Pp. 366. Price 50s.

In this new book the author attempts to interrelate perinatal pathology and physiology. The limitations of our present knowledge on these subjects are well brought out in a critical review of the relevant literature. The author's personal experience is skilfully interwoven, usually in tabulated form. This is not a textbook of descriptive pathology, but the reader will find it a useful reference book which will render the search of the widely scattered literature a much easier task and this is particularly helped by emphasizing in capital letters the more valuable references in the bibliography.

It is to be hoped that this book will stimulate further research on many of its important subject matters: it may for instance help to guide into more useful fields such futile attempts as the study of prematurity in terms of a pathological entity. It is also hoped that the author may overcome in later editions his present difficulties in the classification of some conditions such as are evident when he includes "congenital short oesophagus" among "Hirschsprung's disease and allied disorders."

The publishers are to be congratulated on taking the initiative in this country with the production of a book on a much neglected subject.

MARTIN BODIAN.

Cross Reference.—Dr. Fairfield Smith, who wrote a paper "Urea Clearance Tests" for this Journal (*J. clin. Path.*, 1949, **2**, 266), has published an amplification of his statistical appendix in *Biometrics* (1951, **7**, 185).

Correction.—In "The Question of the Rh Hapten" (*J. clin. Path.*, 1951, **4**, 475), Table VIII, column 2, in the first two sera the upper line refers to the saline control and the second line in each pair refers to lecithin 1:4,000.

J. clin. Path. (1952), **5**, 119.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Combined Active-Passive Re-immunization against Tetanus in Previously Immunized Individuals: Experimental and Clinical Evidence. MILLER, J. J., and RYAN, M. L. (1950). *J. Immunol.*, **65**, 143.

Reactions in animal experiments are described. Similar experiments were repeated in 4 people previously immunized with fluid tetanus toxoid or A.P.T. who were given 0.5 ml. fluid toxoid and 1,500 units tetanus antitoxin (in different limbs) after they had suffered injuries liable to lead to tetanus. In 3 cases the antitoxin was given at the same time as the toxoid, but in the other case, 2 days later. Three out of the 4, including the one in whom administration of antitoxin was delayed, showed a well-marked secondary response to tetanus toxoid; the remaining subject was known from other evidence to give a poor response to tetanus toxoid.

The authors recommend the use of simultaneous active and passive immunization against tetanus for patients who have suffered shock, compound fractures, and massively contaminated wounds at any time after they have been immunized, and for patients suffering less severe wounds 4 years or more after immunization.

[This is an extremely important paper, with an obvious bearing on medical practice: it is to be hoped that the work will be repeated with methods of antitoxin titration that inspire greater confidence. Testing for tetanus antitoxin at the 0.001 unit (U.S.A.) level involves considerable error, and is very liable to give an overestimate of the antitoxin content of a sample. The results for the rabbit that received a mixture of 0.1 ml. fluid toxoid (whose strength is nowhere stated) and 150 units of tetanus antitoxin (stated to be heterologous, presumably refined horse antitoxin, but no information is given) seem to deserve more comment than is given them. Only if the tetanus toxoid contained 1,500 Lf per ml. would the mixture be neutral; if it contained less toxoid (as would ordinarily be expected (some rise in antitoxin titre should have occurred in the rabbit, and later some fall. In no case has an experiment been controlled by injection of antitoxin alone.]

C. L. Oakley.

Bacteriologic Studies of the Newer Antibiotics: Effect of Combined Drugs on Microorganisms. SPICER, S. (1950). *J. Lab. clin. Med.*, **36**, 183.

Penicillin, streptomycin, dihydrostreptomycin, chloramphenicol, aureomycin, and bacitracin were each tested against strains of haemolytic streptococci, *Streptococcus viridans*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *H. pertussis* and pneumococci which were known not to have been in contact with antibiotic drugs. Bacterial sensitivity was measured by the size of the zone of inhibition produced on a seeded plate by a paper disk impregnated with a solution of the antibiotic, and also by determining the number of residual viable organisms to be obtained from the disk by washing with broth.

All antibiotics left a residuum of viable organisms and the number of such organisms was regarded as a better index of sensitivity than the size of the zones of inhibition. By its use, penicillin G was shown to be more potent than penicillin K (as found by clinical experience). Against Gram-positive organisms penicillin was the most effective, bacitracin the next, and aureomycin the least effective. Aureomycin was also the least effective against Gram-negative organisms. Chloramphenicol was the most effective against *H. influenzae* and *H. pertussis*, while streptomycin was the most

active against *K. pneumoniae*. In the case of penicillin, bacitracin, aureomycin, and chloramphenicol the residual organisms were still sensitive to the respective antibiotics, but in the case of streptomycin and dihydrostreptomycin they were resistant.

The effects of penicillin paired with chloramphenicol and aureomycin respectively, and of streptomycin paired with penicillin, chloramphenicol, and aureomycin respectively were determined on the test strains. When organisms were sensitive to both antibiotics, the combined response was additive, but if the organism was sensitive to one of the drugs only the effect of the other was impaired. The synergistic effect is explicable as due to each drug acting on the viable residuum left by the other, while it is suggested that the interfering action is due to the stimulating effect of the ineffective drug on the organism sensitive to the other drug.

M. Lubran.

Sulfonamide Hematuria. Frequency of Injury to the Urinary Tract as Estimated on the Basis of 6,084 Cases Treated with Different Sulfonamide Preparations. [In English.] NISSEN, N. I., AAGAARD, K., and FLINDTHANSEN, E. (1950). *Acta med. scand.*, **138**, 301.

Over 6,000 cases treated with sulphonamides in Frederiksberg Hospital, Copenhagen, are reviewed with reference to the incidence of haematuria. The newer sulphapyrimidine drugs such as sulfadiazine or sulfamerazine, which are excreted more slowly, have a slightly higher tendency to cause renal damage than the older more rapidly excreted types. The lowest incidence was found with "lucosil" (sulphamethylthiodiazole); out of 3,078 cases, haematuria was demonstrated chemically in 2·3%. Children tolerate sulphonamides much better than do adults; the incidence of haematuria increases with age. In no case was the damage to the kidneys permanent.

E. H. Johnson.

The Treatment of Diphtheria Carriers with Tyrothricin. BAGLIONE, L., and DI NOLA, F. (1950). *Minerva med., Torino*, **41**, 1071.

The problem of the diphtheria carrier is an old one, and the methods of treatment numerous.

Tyrothricin is the latest effective agent. In the present investigation 3 ml. of a 1 in 2,000 solution was given twice daily as an aerosol spray. Throat swabs were taken at the end of 4 days and again as necessary until three negatives had been obtained. Of 100 patients only 2 remained refractory, and as many as 54 were rendered negative after only 4 days' treatment. In general, convalescent patients harboured the less virulent organisms, which were easier to remove, while the slower and refractory results occurred in the healthy carrier with highly virulent bacilli. The method is difficult to carry out in younger patients, but appears to be free from toxic effects. *A. Paton.*

Neomycin: Results of Clinical Use in Ten Cases. DUNCAN, G. G., CLANCY, C. F., WOLGAMOT, J. R., and BEIDLEMAN, H. (1950). *J. Amer. med. Ass.*, **145**, 75.

Neomycin is a new antibiotic obtained from *Streptomyces fradiae*. The authors have tested it in 6 cases of pyelonephritis and 4 cases of cystitis, some with complications such as bacteraemia, at the Pennsylvania Hospital, Philadelphia. The causative organisms included *Aerobacter aerogenes*, *Bacterium coli*, paracolon bacilli, *Pseudomonas aeruginosa*, non-haemolytic streptococci, haemolytic staphylococci, and *Proteus*. All these organisms were shown to be completely or partially resistant *in vitro* to penicillin, aureomycin, chloramphenicol, and streptomycin, but in all but one case were sensitive to neomycin (200 units per ml.). Only in the case from which the resistant organism was isolated did the patient fail to improve on treatment, bacteriological evidence of cure being obtained in all the others. Serum and urine assays of the drug were carried out at frequent intervals. Sulphobromophthalein sodium retention tests, phenolsulphonphthalein excretion tests, electro-cardiography, blood and bone-marrow examinations, retinoscopy, audiography, blood urea nitrogen estimations, and urine examinations were carried out before and after treatment and showed evidence of toxicity in only one case. This patient had a persistent elevation of the blood urea nitrogen level, with transient nausea and vomiting and transient impairment of hearing as shown by the audiogram. Dosage varied from 4,498 units (for an infant) to 100,000 units every 6 hours for 4 doses, followed by 50,000 to 100,000 units every 12 hours for 5 to 7 days. In the blood a stable level of 4 to 10 units per ml. was reached in 48 to 72 hours. Maximum urinary levels varied from 26 to 410 units per ml. No relapse has been recorded, the follow-up period ranging from 1 to 4 months. Most strains of *Proteus* and, more especially, of *Pseudomonas* exhibited properties suggesting the potential development of resistance to neomycin. *J. Maclean Smith.*

Clinical Observations on Terramycin. BLAKE, F. G., FRIOU, G. J., and WAGNER, R. R. (1950). *Yale J. Biol. Med.*, **28**, 495.

This paper records the results of treatment with terramycin of 15 patients with various infections. Terramycin hydrochloride was given orally in all cases. Various dosage schemes were employed, but treatment usually consisted of an initial dose of 1 g. repeated 4-hourly for 3 doses, followed by 3 to 6 g. a day at 3- or 4-hourly intervals. Among the conditions in which good results were obtained were pneumococcal lobar pneumonia and infections due to staphylococci, *Streptococcus viridans*, *Strep. faecalis*, some strains of *Bacterium coli*, *Bacillus mucosus capsulatus*, *Proteus vulgaris* and Vincent's organisms. In 2 cases of subacute bacterial endocarditis the response was good, but relapse occurred as soon as treatment was stopped. The only toxic effects encountered were nausea, vomiting, and diarrhoea, which occurred in 12 of the 15 cases, but were troublesome in only 3. *A. W. H. Foxell.*

Enhancement of the Action of Streptomycin. REES, R. J. W., and ROBSON, J. M. (1950). *Science*, **112**, 790.

The authors describe the experimental treatment of tuberculous lesions of the cornea in rabbits and mice with combinations of streptomycin and various other drugs, the results of which confirm the statement of Woody and Avery (*Science*, 1948, **108**, 501), that the action of streptomycin against the tubercle bacillus can be enhanced by combining it with potassium iodide. They found that this enhancement was more definite in advanced caseous tuberculosis than in very early lesions. Their findings also confirm the work of Bavin (*J. Pharm. Pharmacol.*, 1949, **1**, 790), who showed that the effect of *p*-amino-salicylic acid (2% in the diet) was not enhanced by the addition of potassium iodide. They report that treatment with either streptomycin alone or with *p*-amino-salicylic acid alone was more effective than treatment with a streptomycin *p*-aminosalicylate compound. *A. W. H. Foxell.*

Studies on the Administration, Absorption, Distribution and Excretion of Aureomycin in Children. With Observations Concerning Tolerance, Dosage Schedules and Certain Therapeutic Indications. WHITLOCK, C. M., HUNT, A. D., and TASHMAN, S. G. (1950). *Pediatrics*, **6**, 827.

This paper records observations on the administration, absorption, distribution, and excretion of aureomycin in 150 patients, ranging from newborn infants to adults. The authors' conclusions may be summarized as follows.

Aureomycin in doses of approximately 11 mg. per kg. body weight given orally at 4-hour intervals is well tolerated by the majority of infants and children. Satisfactory serum levels are obtained with this dosage. Single doses larger than 11 mg. per kg. body weight do not produce significantly higher serum levels. The incidence of vomiting among 48 children on such a dosage schedule was 19% (9 cases). Gastro-intestinal tolerance was increased when aluminium hydroxide gel or 3 to 4 oz. (85 to 114 ml.) of milk were given following each dose. When the intravenous route is employed the recommended dosage is 6·6 mg. per kg. body weight every 12 hours. The intramuscular route is unsatisfactory as it causes intense local pain and frequent febrile reactions. Rectal administration was tried in 3 cases but was found to be unreliable and often painful.

The peak serum level is reached somewhere between 1 and 4 hours after ingestion. This level is low but is maintained until 6 to 8 hours after ingestion. On the other hand, after intravenous administration there is a rapid rise and fall of the aureomycin level in the serum. The authors suggest that aureomycin is distributed rather uniformly throughout the body, but that it does not enter the erythrocytes. The fact that carinamide does not seem to affect the slope of the aureomycin serum level curve suggests that tubular excretion plays a negligible role, and that renal excretion is glomerular alone.

A. W. H. Foxell.

Treatment of *Hemophilus influenzae* Meningitis with Chloramphenicol and Other Antibiotics. MCCRUMB, F. R., HALL, H. E., IMBURG, J., MERIDETH, A., HELMHOLD, R., and BASORA Y DEFILLO, J. (1951). *J. Amer. med. Ass.*, **145**, 469.

The effect of chloramphenicol alone in the treatment of 12 cases of *Haemophilus influenzae* meningitis occurring in children whose ages ranged from 5 months to 5 years (mean 2·4 years) is described. Experiments on mice demonstrated that while streptomycin, aureomycin, and chloramphenicol varied but slightly in protective power, terramycin was inferior and penicillin ineffective against *H. influenzae*. Chloramphenicol was chosen for use because of ease in administration, low toxicity, and a stability which simplified bio-assay.

In the present series chloramphenicol was given orally with an initial dose of 750 mg.

followed by 250 mg. 4-hourly. The average duration of fever after instituting therapy was 2·3 days. There was rapid improvement with return to consciousness in on the average 1·3 days. All patients recovered and in one only was there any neurological residue, a patient in whom treatment had begun only on the seventh day, after terramycin had failed. In 5 cases the concentration of chloramphenicol in the cerebrospinal fluid averaged 13·2 μg., roughly half that in the blood.

The authors state that they have insufficient facts on which to recommend an optimum concentration, but with the dosage here employed no toxic manifestations were encountered. Oral administration presented no serious difficulty but it is stated that rectal administration of 500 mg. aids in the oral treatment. The mean duration of treatment was 8 days and the total received by one patient 10·4 g. The authors express the opinion that at present chloramphenicol is the antibiotic best suited to deal with infection by any of the Gram-negative group of pathogens. *Joseph Ellison.*

A Note on 40 Cases of Typhoid or Paratyphoid Fever in Infants Treated and Cured with Chloramphenicol. MARIE, J., SERINGE, P., SEE, G., and MARLAND, P. (1951). *Sem. Hôp. Paris*, **27**, 630.

Although typhoid fever usually runs a benign course in infants, the mortality before the days of modern antibiotics was certainly not zero. The authors treated 40 cases of typhoid or paratyphoid B between September, 1949, and September, 1950, with chloramphenicol. All these patients recovered rapidly. Convalescence was established always within a week and generally in 4 days. No complications occurred after the inception of treatment, but 2 cases of typhoid encephalitis and one of typhoid osteomyelitis were cured completely when the antibiotic was employed. Only one patient suffered relapse, the symptoms returning twice, with positive blood cultures, after apyrexial intervals of a fortnight; both relapses responded rapidly to renewed chemotherapy. It is suggested that these episodes were due to the release of organisms from the gall-bladder where they had been shielded from the impact of the antibiotic. On the second occasion an attempt at medical drainage was made with posterior pituitary extract and adrenaline. Small infants received 50 to 100 mg. per kg. body weight, and larger infants 1·5 to 2 g. of chloramphenicol daily by mouth. No difficulty was found in getting the capsules swallowed. They were softened in boiled water and then placed on the tongue immediately before the presentation of a feed. The authors deprecate the use of a "loading dose" and, indeed, recommend the use of half doses for the first 2 days of treatment. It is to this routine that they attribute the freedom from side effects in the series. The chemotherapy must be maintained for an adequate period which is considered to be between 1 and 2 weeks. No evidence was found to support the suggestion that the use of chloramphenicol interferes with the proper development of antibodies to the infection. *T. A. A. Hunter.*

Chloramphenicol in Progressive Dosage in the Treatment of Hypertoxic Typhoid Fever. FARINAUD, M. E., and PORTES, L. (1951). *Pr. méd.*, **59**, 3.

From Indochina the authors report 12 cases of typhoid treated with chloramphenicol: 4 fever patients were treated with the orthodox initial dose of 50 mg. per kg. body weight, followed by similar daily maintenance doses: they died with signs of vascular collapse or cerebral irritation. In further cases where the length of the history or the hypertoxic state of the patient appeared to warrant it, a much lower initial dosage scheme was employed, 15 mg. per kg. in four divided doses during the first day, with a gradual daily increase of 0·5 to 1 g. until the patient became afebrile, followed by maintenance of this dosage, usually about 50 to 60 mg. per kg. for 7 days. Symptomatic treatment with analeptics and deoxycortone was given when indicated. This regimen did not, in the cases reported, delay defervescence, which occurred on the 6th or 7th day of treatment. *W. G. Harding.*

Dysentery Due to Sulfonamide-resistant *Shigella sonnei* Controlled with Chloromycetin. COOPER, M. L., and KELLER, H. M. (1950). *Amer. J. Dis. Child.*, **80**, 911.

Shigella sonnei was isolated from the faeces of 5 patients in a children's hospital who had become acutely ill with fever and diarrhoea. The source of infection was traced to a symptomless carrier among the nursing staff who had had a mild attack of diarrhoea 3 months before the outbreak: 4 of the children and the carrier were treated initially with sulphadiazine by mouth. The therapeutic response to this drug was poor and *Sh. sonnei* persisted in the faeces for an average of 12 days. The other child was given chloramphenicol by mouth and rectally, and after 2 days' therapy the faeces were clear of shigella. *In vitro* sensitivity tests revealed that

the infecting strains were highly resistant to sulphadiazine and sensitive to chloramphenicol. Accordingly, the cases which were refractory to sulphadiazine were treated with chloramphenicol, and within 4 days of the change in therapy *Sh. sonnei* was eliminated permanently from the faeces.

Groups of mice were infected with two of the epidemic strains and were treated with sulphadiazine, sulphapyrazine, or chloramphenicol. The mortality rate was high in the groups treated with the sulphonamide drugs, and was negligible in those given chloramphenicol.

G. B. Forbes.

A Fatal Case of Aplastic Anemia following Chloramphenicol (Chloromycetin) Therapy. RICH, M. L., RITTERHOFF, R. J., and HOFFMANN, R. J. (1950). *Ann. intern. Med.*, **33**, 1459.

A case is reported of a patient who was treated for 3 months for pyuria by administration of 0·25 g. of chloramphenicol every 8 hours. He developed a severe anaemia and decrease of blood platelets with an extensive purpuric rash. After death the main pathological changes were found in the lungs which were very oedematous, the bronchi being filled with coagulated protein and erythrocytes. Focal necroses were present round many bronchi and bronchioles. There was a marked reduction of haemopoietic elements in the sternal marrow. Only small amounts of other drugs were given and these were not of a kind likely to cause such a condition, which the authors attribute to the nitro-benzene group in the chloramphenicol molecule.

V. I. Woolley.

A New Method for Treatment of Brucellosis. HERRELL, W. E., and BARBER, T. E. (1950). *J. Amer. med. Ass.*, **144**, 519.

The authors, after reviewing briefly the antibiotics and chemotherapeutic agents which have been used, either singly or in combination, for the treatment of human brucellosis and the experimental disease in mice, give a detailed account of 35 cases in which the patient was given, concurrently, aureomycin hydrochloride and dihydrostreptomycin sulphate. The diagnosis in 21 cases was confirmed by blood culture, the organisms identified being: *Brucella abortus* (9), *Br. melitensis* (7), and *Br. suis* (4), with one unclassified organism. In 4 cases only localized lesions were present, from which *Br. abortus* was grown, once (spine) and *Br. suis* three times (thigh sinus, inguinal lymph node, lung tissues). Ten cases were diagnosed on the basis of the symptomatology and presence of high agglutinin titres. Scheduled treatment was: aureomycin orally 1 g. daily in 6-hourly doses, and dihydrostreptomycin 1 g. intramuscularly morning and evening, for 12 to 14 days; in cases with localized lesions the treatment was continued for 28 days, the dose of streptomycin being halved after the 14th day.

Temperature and symptoms subsided in every case 48 to 72 hours after starting treatment, and no relapse was observed on following-up for 9 to 19 months, with the exception of one patient taking aluminium hydroxide gel (which apparently interferes with the absorption of aureomycin). There were no signs of toxicity or of 8th nerve involvement. The symptomatology, diagnosis, and treatment are clearly and thoroughly described, one case resembling primary atypical pneumonia closely. All varieties of brucellosis seemed to respond equally well.

[These remarkably successful results will no doubt lead to further trials of combinations of antibiotics in infections so far resistant. It would have been of interest to know the method of culture employed, but this is not stated.]

L. J. M. Laurent.

Incidence of Toxoplasma Infection in North-west England. Transmission of Antibody from Mother to Foetus. MACDONALD, A. (1950). *Lancet*, **2**, 560.

The incidence of toxoplasmosis in north-west England was investigated by testing 12 children with clinical signs (chorio-retinitis and cerebral calcification), 88 with doubtful signs, and a control series of 350 cases (150 blood donors, 100 normal children under 10 years, and 100 other adults serologically).

In 10 of the 12 clinically diagnosed cases, 6 of the 88 doubtful cases and 13 of the controls, the complement-fixation reaction for toxoplasmosis was positive. Among the 100 normal children under 10 years there were no positive reactions, so that the incidence of symptomless infection in the adults in the control series was 5%. This means that it is impossible to diagnose congenital toxoplasmic infection in a child solely by examination of the maternal blood.

Re-examination of mothers' sera at intervals showed no detectable loss of complement-fixing antibody during periods of as long as 12 months, but whether this was due to continued infection or merely to a continuing stimulus to antibody formation is not known. The findings of Sabin and Feldman were confirmed; these workers had shown that antibodies from mothers who had given birth to infected infants were transferred in subsequent

pregnancies to infants who remained uninfected and whose transferred antibodies disappeared after 4 to 5 months.

It is suggested that possibly maternal antibody is protective to the foetus, and that only those mothers infected during pregnancy are likely to give birth to infected infants. This would mean that subsequent children are likely to be born uninfected. *Wilfrid Gaisford.*

Q Fever in Great Britain. The Causative Agent. STOKER, M. G. P. (1950). *Lancet*, **2**, 616.

Two strains of *Rickettsia burnetii* isolated in Great Britain, one from a patient with Q fever, the other from milk, were compared in the laboratory with a standard Italian strain. The British strains were similar in shape, size, and pathogenicity for the guinea-pig. The milk strain was more readily adapted to growth in the yolk-sac of fertile hens' eggs, and appeared more pathogenic for guinea-pigs than the human strain. There was complete cross-protection with all three strains in guinea-pigs. Antisera against the British strains reacted with antigens from the Italian strain, but antigens from the British strains would not react until they were prepared from strains which had become adapted to the egg. A change in antigenic structure during egg adaptation is suggested as the explanation.

J. E. M. Whitehead.

Inclusion Disease or Generalized Salivary Gland Virus Infection. SMITH, M. G., and VELLIOS, F. (1950). *Arch. Path.*, **50**, 862.

In this disease the nucleus of the cell is enlarged and the cytoplasm increased in amount. Large intranuclear inclusions surrounded by a clear halo and lying within a distinct nuclear membrane, containing one or more dense chromatin masses, are pathognomic of the disease. These lesions, for the most part, occur in the viscera and particularly in the lungs. They have also been seen in the kidney, liver, spleen, and other organs, and are accompanied by symptoms of blood dyscrasia or hepatic damage: in about one-third of the known cases there was an association with a previous attack of whooping-cough. In all, 69 instances of the disease have been reported, occurring, for the most part, in the newborn or very young, although one instance of the disease in a child of 13 years is known.

It is probable that the disease is a virus infection because the characteristic lesions closely resemble those seen in salivary gland virus infections in rodents. *R. Hare.*

The Etiology of Epidemic Pleurodynia: a Study of Two Viruses Isolated from a Typical Outbreak. WELLER, T. H., ENDERS, J. F., BUCKINGHAM, M., and FINN, J. J. (1950). *J. Immunol.*, **65**, 337.

During an epidemic of acute pleurodynia which occurred in Massachusetts in the summer of 1947 attempts to isolate the causal agent were unsuccessful. Since then the isolation of strains of a virus pathogenic to infant mice from cases diagnosed as " non-paralytic poliomyelitis " has been reported, and laboratory infections by these " Coxsackie " strains have been observed to be clinically similar to epidemic pleurodynia. The authors have therefore re-examined the material obtained during the 1947 pleurodynia epidemic in an attempt to define its relationship to the Coxsackie group of viruses.

Throat washings had been collected in horse-serum saline and stored on dry ice. Two of the 6 throat washings examined contained an agent pathogenic for suckling mice. Nine pairs of sera collected from patients during the 1947 epidemic were then selected at random and examined for neutralizing antibodies.

The results of these experiments suggest that a close antigenic relationship exists between the agents responsible for epidemic pleurodynia and the Coxsackie group of viruses, although their pathogenic potentialities differ widely, as do those of the various Coxsackie strains. (Reference is made, in an addendum, to the report by Findlay and Howard (*Brit. med. J.*, 1950, **1**, 1233; *Abstracts of World Medicine*, 1950, **8**, 310), published after completion of the present work, in which similar conclusions were reached.)

BIOCHEMISTRY

The Amino-acid Pattern in Human Foetal and Maternal Plasma at Delivery. CRUMPLER, H. R., DENT, C. E., and LINDAN, O. (1950). *Biochem. J.*, **47**, 223.

The authors investigated the concentration of α-amino nitrogen and of individual amino-acids in the foetal and maternal blood of 9 parturient women at University College Hospital, London.

Foetal blood was taken from the placental side of the cut umbilical vein immediately after tying; maternal blood was obtained from the antecubital vein immediately afterwards. Heparin was used as anticoagulant.

Analysis of α-amino nitrogen was made on either plasma or plasma ultrafiltrate by the ninhydrin-CO_2 method of Hamilton and Van Slyke. The concentrations of individual

amino-acids were determined chromatographically on solutions derived from plasma freed of protein by ultrafiltration and electrolytically desalted.

The concentration of α-amino nitrogen was found to be raised in foetal blood compared with maternal blood, the ratios varying between 3:1 and 1.6:1 in non-toxaemic cases and between 1.4:1 and 1:1 in 4 cases of toxaemic pregnancy. Chromatographic analysis showed that individual amino-acids were all concentrated to approximately the same extent by the placenta, which thus appears to act in such a way as to aid foetal synthesis of protein.

HAEMATOLOGY

Activity of Plasma Labile Factor in Disease. STEFANINI, M. (1951). *Lancet*, **1**, 606.

The plasma labile factor is a necessary constituent of the mechanism for the proper formation of thrombin. It has been called by various workers "component A," "factor V," and "plasma Ac globulin". The labile factor has certain well defined characteristics which enable its concentration in the plasma to be determined. The author has devised a quantitative method of estimating the factor, and used it in studying the importance of depletion of the labile factor in determining the prolonged prothrombin time in various diseases.

Blood samples from 187 persons were examined, 20 being healthy subjects, the rest having a prolonged prothrombin time or active bleeding: 15 cases of hypoprothrombinaemia due to dicoumarol and 15 post-operative cases were also investigated. Prothrombin activity, prothrombin concentration, and labile-factor activity were determined in each case. (References to the methods used are given.) In hepatic disorders the labile-factor activity is normal in obstructive jaundice, but decreased in liver dysfunction. Large doses of vitamin K did not restore the activity of the factor even though the prothrombin activity returned to normal, suggesting that vitamin K is not required for the synthesis of the factor.

In the group of haemorrhagic conditions the labile-factor activity was normal in those which were due to a vascular abnormality and in idiopathic and allergic thrombocytopenia. But in all cases of symptomatic thrombocytopenia studied there was a striking depletion in labile-factor activity. Although no change in the activity of the factor was found in haemophilia it was noted that there was a prolonged clotting time and decreased prothrombin activity a week preceding and a week following a spontaneous haemorrhage. In hypoprothrombinaemia of the newborn labile-factor activity was normal, but the prothrombin activity was decreased significantly and was lower than the prothrombin concentration, suggesting a further plasma factor. No change in the activity of the labile factor was found during dicoumarol therapy, contrary to the findings of other workers: this may be explained by the different methods used. In terminal carcinoma the labile-factor activity and prothrombin activity and concentration all fell, the fall being roughly parallel with that in serum protein level, as in liver dysfunction. In the post-operative cases the labile-factor activity had decreased to about 40% of normal by the third day.

In his discussion the author points out that although it appears that the liver is the main source of the factor, it may not be the only source, as no liver dysfunction is demonstrable in those blood dyscrasias showing a low labile-factor activity. The importance of fresh blood in the treatment of haemorrhage in those conditions with a low labile-factory activity is obvious. A low labile-factor activity without diminished prothrombin activity is seldom found.

R. F. Jennison.

The Anemia of Thermal Injury. I. Studies of Pigment Excretion. JAMES, G. W., PURNELL, O. J., and EVANS, E. I. (1951). *J. clin. Invest.*, **30**, 181.

The excretion of pigments in urine and faeces was studied in a series of patients with burns of varying severity. Haemolysis, as judged by increased faecal urobilinogen excretion compared with total circulating haemoglobin, was present in all cases in the first few days after injury. It was greatest in third-degree burns involving more than 20% of the body surface. A remarkable increase was noted in urinary urobilinogen on the third day after injury; this they consider an indication of early hepatic dysfunction. The anaemia associated with burns is thought to be due in part to haemolysis and in part to dyshaematopoiesis dependent on disordered liver function. Oral aureomycin reduces both the faecal and the urinary urobilinogen to small amounts owing to a sterilizing action on the faecal flora. The authors question whether the petroleum-ether-soluble, Ehrlich-reacting substances found after oral aureomycin administration are urobilinogens or whether they are entirely different compounds.

Janet Vaughan.

The Anemia of Thermal Injury. II. Studies of Liver Function. JAMES, G. W., PURNELL, O. J., and EVANS, E. I. (1951). *J. clin. Invest.*, **30**, 191.

In a study of pigment excretion following severe thermal injury the authors found an increased excretion of urinary urobilinogen, which they interpreted as evidence of liver damage. They therefore undertook an investigation of certain liver-function tests in burned patients, none of whom received sulphonamides, tannic acid, or other escharotic therapy. They found that minor to extensive third-degree burns showed an early impairment of liver function as judged by changes in (1) bromsulphalein retention; (2) cephalin–cholesterol flocculation; (3) thymol turbidity; (4) prothrombin time; (5) total protein and albumin–globulin ratio; (6) serum bilirubin level; and (7) urinary urobilinogen content. The most constant changes were found in this last and in the albumin–globulin ratio. Necropsy material in 5 fatal cases showed no constant histopathological change, but there was evidence of fatty infiltration, cloudy swelling, increased pigments in the reticulo-endothelial cells, focal necrosis, and congestion in the liver substance.

The authors suggest that attention should be directed to the prevention as well as treatment of liver dysfunction in severe burns.

Janet Vaughan.

Direct Splenic Arterial and Venous Blood Studies in the Hypersplenic Syndromes before and after Epinephrine. WRIGHT, C. S., DOAN, C. A., BOURONCLE, B. A., and ZOLLINGER, R. M. (1951). *Blood*, **6**, 195.

This is one of a series of studies from the Ohio State University on the various phases of the hypersplenic mechanism. The reservoir function of the spleen may be pathologically increased and therapeutically decreased, with a reversal of the formed elements of the blood sequestered in its pulp-sinusoidal spaces. Figures were obtained from: (1) blood volume and peripheral blood cell differential studies before and after subcutaneous adrenaline injections. These, in cases of chronic myelogenous leukaemia, demonstrate that the degree of splenic enlargement is a direct function of the abnormal delivery and storage of the granulocytic cells. With treatment, as granulopoiesis in the marrow becomes less dominant, delivery of these cells to the circulation decreases, erythropoiesis increases reciprocally, normal delivery of erythrocytes to the blood is resumed, and the spleen again returns to normal size. In a case of thrombocytopenic purpura, following adrenaline the platelet count rose, transitorily, from 20,000 to 100,000 per c.mm. with no parallel erythrocyte or leucocyte increase. (2) Splenic arterial and venous blood obtained at laparotomy before and after adrenaline injection into the artery. The subjects were 19 patients with primary hypersplenism (thrombocytopenic purpura, hereditary spherocytosis, and splenic panhaematopenia) and 17 with some constitutional disease involving the spleen. The primarily involved blood-cell elements characteristically entered the spleen in larger numbers than were found leaving this organ. Further, the involved elements tended to be increased in the splenic venous blood after adrenaline-induced contraction. After the surgical removal of all splenic tissue in hypersplenic syndromes, the subcutaneous injection of adrenaline failed to affect significantly the re-established, relatively stable, circulating cellular equilibrium in the blood.

A comparison of the pre-operative adrenaline test with the adrenaline test at laparotomy, made in 2 selected cases, showed that effective cellular mobilization, as interpreted directly from several peripheral blood counts, paralleled closely the direct evidence from splenic venous blood samples.

These findings favour the hypothesis that spleen-withholding rather than bone-marrow suppression of blood cells plays the major part in many of the hypersplenic cytopenic states.

Harold Caplan.

Aplastic Anaemia. BOON, T. H., and WALTON, J. N. (1951). *Quart. J. Med.*, **20**, 75.

The authors review 25 cases of aplastic anaemia seen during the last 10 years at the Royal Victoria Infirmary, Newcastle, and give detailed case histories of 6 which were cured by the repeated transfusion of packed erythrocytes. Neoarsphenamine had been given to one patient, gold injections to 2, but in the other 3 cases no drug was incriminated as the causative agent. Comment is made on the psychological strain of the repeated transfusions on the patient and the doctor; the frequency of reactions despite careful cross-matching; the value of polythene tubing in reducing phlebothromboses and venous spasm in restless nervous patients; and the routine use of antibiotics to prevent infective complications. In 2 cases intrasternal injections of bone marrow from donors of the same genotype were given with benefit.

Ernest T. Ruston.

The Treatment of Polycythemia Vera with Radioactive Phosphorus. WISEMAN, B. K., ROHN, R. J., BOURONCLE, B. A., and MYERS, W. G. (1951). *Ann. intern. Med.*, **34**, 311.

This is a report of experience gained in treating 108 cases of polycythaemia vera with radioactive phosphorus during a period of 10 years. The results are contrasted with those in a series of 30 cases treated by the ordinary conventional methods. The aetiology and therapeutic considerations in polycythaemia are considered, and then the method of internal radiation therapy is described. The effect on the symptoms, signs, and haematological picture in this group of cases is described, and the causes of death are discussed. There is a detailed description of the method of using radioactive phosphorus in the treatment of polycythaemia vera. The authors conclude that the method is haematologically sound, effective, inexpensive, and convenient for use. They think that with adequate controls this method of treatment is safe.

John F. Wilkinson.

Haemophilia Associated with Normal Coagulation Time. MERSKEY, C. (1951). *Brit. med. J.*, **1**, 906.

The most important laboratory test for haemophilia is the prolongation of the clotting time, but there seems little doubt that the test may be normal in very mild haemophiliacs and in ordinary cases in remission. In haemophilia, prothrombin is not used up in normal amounts in the clotting process. Thus, haemophilic serum may contain as much prothrombin as does the plasma. [In the clotting of normal blood nearly all the prothrombin is turned into thrombin.] Antihaemophilic globulin, which is found in normal blood, will hasten the clotting of haemophilic blood. The latter does not, of course, contain this globulin, and its absence can be proved by adding the suspected blood in minute quantities to haemophilic blood; there is no shortening of the clotting time. These three tests can be used in the diagnosis of haemophilia.

This paper describes the clinical histories of 8 families in which cases occurred with normal or only mild prolongation of the clotting time. Symptoms might be mild or severe; patients with mild symptoms lead fairly normal lives. Most of the patients, however, had had at least one severe bleeding bout, especially when teeth were extracted. Minor scratches gave little trouble, and only followed severe injury; thus ankylosed joints were infrequent. The author rightly stresses that the finding of a normal coagulation time may give a false sense of security, especially with regard to operations. Cases linked by family connexions were similar as regards both the clinical picture and the laboratory findings. *Paul B. Woolley.*

Haemophilia in the Female. ISRAËLS, M. C. G., LEMPERT, H., and GILBERTSON, E. (1951). *Lancet*, **1**, 1375.

The case is described of a young woman aged 24 whose parents belonged to haemophilic but unconnected families. Her father and his brother, and her mother's brother, were known haemophiliacs. Her sister's son was also a haemophiliac and was found by the authors to show the typical coagulation defect of this disease.

The patient had been known to bruise easily since childhood. She came under investigation on the present occasion as a primipara who had gone to full term but had developed severe postpartum haemorrhage. Repeated investigation over a period of about 9 months provided convincing laboratory evidence of the coagulation defect of haemophilia. The platelet count was uniformly normal; the coagulation time was invariably prolonged; the accelerated coagulation time (oxalated plasma) was always greatly increased; and prothrombin consumption was very much delayed. The plasma prothrombin time was normal, the labile factor was shown to be present, and there was no demonstrable anticoagulant in the patient's plasma. Adequate controls were included at all stages in the investigation, and there is no doubt that the case described satisfies all the established requirements necessary for the diagnosis of haemophilia.

A. Brown.

The Mechanism of Hemolysis in Paroxysmal Cold Hemoglobinuria. I. The Role of Complement and its Components in the Donath–Landsteiner Reaction. JORDAN, W. S., PILLEMER, L., and DINGLE, J. H. (1951). *J. clin. Invest.*, **30**, 11.

Studies of sera from 2 patients with paroxysmal cold haemoglobinuria (PCH) have shown that large amounts of complement may be necessary for haemolysis in the Donath–Landsteiner reaction. By using an adequate amount of complement, the haemolysins from both patients were found to be stable at 62° C.

An excess of complement is necessary for antibody titration, since a reciprocal relationship exists between the amount of complement present and the antibody titre. Agglutination by

antiglobulin serum of erythrocytes sensitized in dilutions of PCH serum provides another measure of antibody level, the titres obtained being comparable to those found by haemolysin titration in the Donath–Landsteiner reaction.

Complement is essential in both the cold and warm phases of this reaction, and there is a reciprocal relationship between the amounts of intact complement required in these two phases. The erythrocyte–PCH antibody system is unique in that it requires complement for antibody fixation as well as for subsequent haemolysis.

The PCH haemolytic system is unusual in another respect, because all components of complement are not necessary for haemolysis. Haemolysis occurs in the absence of C/1 and C/3. Haemolysis does not occur when C/4 is missing in the cold phase, or when C/2 is missing in the warm phase.

Since only two components of complement are required for haemolysis in PCH, titration of complement in a serum by the sheep cell-amboceptor system, which requires all four components, may not measure the capacity of that serum to produce haemolysis with PCH antibody.—[Authors' summary.]

MORBID ANATOMY AND HISTOLOGY

The Renal Appearances in the Microscopic Form of Periarteritis Nodosa. WAINWRIGHT, J., and DAVSON, J. (1950). *J. Path. Bact.*, **62**, 189.

The present paper is an extension of an earlier publication (Davson, Ball, and Plant, *Quart. J. Med.*, 1948, **17**, 175) in which the authors described the renal lesions in cases of polyarteritis nodosa. They now record in detail the renal changes in 4 further cases of the disease and 2 probable cases, stressing in particular the morbid anatomy, which they consider to be distinctive. The kidneys were enlarged, smooth, and congested. The characteristic feature was the presence of visible glomeruli as opaque grey spots. Microscopically the kidneys in all cases showed an "explosive" glomerulitis with necrosis, cellular infiltration, and pericapsular infiltration. Polyarteritis nodosa was found in 4 cases, but its extent varied from the involvement of many organs to that of a few arteries only. They feel justified in including their 2 further cases because of this variability and because in all other respects they were exactly similar. They suggest that, in middle-aged patients who die of a fulminating acute nephritis without hypertension and whose kidneys show the naked eye lesions which they describe, a presumptive diagnosis of polyarteritis nodosa can be made in the post-mortem room.

The Relationship of the Degree of Coronary Atherosclerosis with Age, in Men. WHITE, N. K., EDWARDS, J. E., and DRY, T. J. (1950). *Circulation*, **1**, 645.

The authors examined necropsy specimens of the heart in 600 male subjects who had died from various causes at ages ranging from 30 to 89 years, 100 being examined in each decade. Cross-sections were made and carefully examined at 3-mm. intervals throughout the length of the main stem and the anterior descending and circumflex branches of the left coronary artery, and of the main stem and the marginal and posterior descending branches of the right. The degree of coronary arteriosclerosis present, if any, was assessed on a basis of four grades ranging from minimal (grade 1) to complete closure (grade 4) in each main stem and in the proximal, middle, and distal segment of each branch. It was found that the average degree of sclerosis present increased steadily with the subject's age from 30 to 60 years, but decreased slightly in those over 60 years of age. Sclerosis was most marked in the proximal segment of the anterior descending branch, reaching grade 3 in 11% of those dying between 50 and 60 years. Sclerosis was least marked in the small marginal branch of the right coronary; in the remaining arteries, including the right coronary and its posterior descending branch, the degree was approximately equal. Hypertension, as evidenced by cardiac enlargement, had little effect on the amount of sclerosis, which was only 10% greater in subjects with hearts which weighed 450 g., or more, than in those which weighed less.

C. W. C. Bain.

Primary Pulmonary Hypertension and Related Vascular Changes in the Lungs. SPENCER, H. (1950). *J. Path. Bact.*, **62**, 75.

The scanty literature on primary pulmonary hypertension is here reviewed and 6 new cases are described. Details are provided of the changes in the main pulmonary arteries and in their ultimate muscular branches.

J. clin. Path. (1952), **5**, 129.

THE VITAMIN B_{12} CONCENTRATIONS OF SERUM AND URINE OF NORMALS AND OF PATIENTS WITH MEGALOBLASTIC ANAEMIAS AND OTHER DISEASES

BY

D. L. MOLLIN AND G. I. M. ROSS*

From the Department of Pathology, Postgraduate Medical School of London

(RECEIVED FOR PUBLICATION SEPTEMBER 28, 1951)

Until recently deficiency of vitamin B_{12} in man could only be recognized by finding the characteristic clinical and haematological signs of the deficiency and by demonstrating that these signs were cured or relieved by treatment with the vitamin. Changes in the vitamin B_{12} content of the tissues could only be inferred from the responses of patients to treatment.

The recent introduction of highly sensitive methods of microbiological assay has made it possible to measure the actual vitamin B_{12} content of body fluids. *Euglena gracilis* var. *bacillaris* is the most sensitive organism for microbiological assay (Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes, 1949; Ross, in the press), and the purpose of this paper is to report the results of assays with this organism and to demonstrate the value of the assay method in the study of megaloblastic anaemias.

The serum and urinary vitamin B_{12} concentrations of patients with pernicious anaemia have been found to be much lower than those of both normal subjects and of patients suffering from other diseases. The concentrations found in patients suffering from megaloblastic anaemias other than pernicious anaemia varied. In some the concentrations were as low as those found in pernicious anaemia, but in others the vitamin B_{12} concentrations were normal.

Materials and Methods

Serum.—Blood was obtained by venepuncture, using a dry syringe, and was allowed to clot in a sterile glass container at 37° C. The serum was removed after one hour at this temperature and kept frozen until assayed. Assays were usually made within three days of collection.

Urine.—Twenty-four hourly collections were made, using toluene as preservative. In most instances each sample was stored at 4° C. immediately after being passed. Assays were usually made within three days of collection.

Method of Assay.—The vitamin B_{12} concentration in the serum and urine was measured by a modification of the assay method introduced by Hutner *et al.* (1949), in which the green alga *Euglena gracilis* was used as the test organism. Details of the technique will be published in the next issue of this journal. As little as 10 $\mu\mu$g. per ml. of vitamin B_{12} are detectable in serum and urine with this method.

* In receipt of a grant from the Medical Research Council.

Material with vitamin B_{12}-like growth activity for *Euglena gracilis* may occur in serum either free or in combination with some substance, probably protein (Ross, 1950). The combined form is microbiologically inactive and it is necessary to free the vitamin from combination before it can be assayed. Heating dilutions of the test fluids at 100° C. has been used to accomplish this.

The values given below have been expressed as either " uncombined (or free)," "combined," or " total." Uncombined vitamin B_{12} is the amount detected by assay after heating in a 56° C. water bath for 30 minutes. (Heating at 56° C. does not liberate vitamin B_{12} from combination but is done to reduce contamination.) Total vitamin B_{12} is the amount detected after heating in a 100° C. water bath for 15 minutes, and combined vitamin B_{12} is the difference between the total and the uncombined concentrations.

Blood Counts.—Venous blood collected into ammonium and potassium oxalate mixture was used throughout.

Red Cell Counts.—These were made at a dilution of 1 : 200, using Bürker or Neubauer counting chambers. A minimum of 2,000 cells were counted for each count.

Haemoglobin.—Haemoglobin was estimated by various modifications of the alkaline-haematin or oxyhaemoglobin methods. Readings were made either with a photoelectric colorimeter or with the M.R.C. grey-wedge photometer.

Packed Cell Volume.—This was measured by Wintrobe's method. The tubes were centrifuged at 3,000 r.p.m. for 30 minutes.

Reticulocyte Counts.—These were made on dry films after staining red cell suspensions with 0.4% cresyl blue for 30 minutes at 37° C. The reticulocytes present in at least 1,000 cells were counted.

Results

The Vitamin B_{12} Concentration of Serum.—The total and combined serum vitamin B_{12} concentrations of 65 normal subjects aged between 15 and 40 years are given in Table I. In the same table are included the levels of 63 patients with various conditions other than megaloblastic anaemia, and of 43 patients with pernicious anaemia in mild, moderate, or severe relapse. It can be seen that the differences between the total and the combined serum vitamin B_{12} concentrations of each group are small, indicating that most of the vitamin in these sera is in the combined form.

Normals and Patients with Conditions other than Megaloblastic Anaemia.—The total serum vitamin B_{12} concentrations of the normal group ranged from 100 to 720 $\mu\mu$g. per ml., with a mean of 358 $\mu\mu$g. per ml. The combined concentration ranged from 100 to 620 $\mu\mu$g. per ml., with a mean of 329 $\mu\mu$g. per ml. The mean vitamin B_{12} serum concentration found in patients with conditions other than megaloblastic anaemias was not significantly lower than in the normal subjects. The individual level of a few patients in this group was, however, low (Table II).

Patients with Pernicious Anaemia.—The serum vitamin B_{12} concentrations of patients suffering from pernicious anaemia in relapse were very much lower than the values found in the other groups and ranged from less than 16 to 105 $\mu\mu$g. per ml. The mean concentration given in Table I for this group of patients is certainly too high, because in 24 instances the concentrations were too low for definite figures to be obtained. These results were not included in those from which the mean was calculated. In 11 of these sera containing only traces of vitamin B_{12} the amount present was less than 40 $\mu\mu$g. per ml.; in 13 it was less than 20 $\mu\mu$g. per ml.

TABLE I

TOTAL AND COMBINED SERUM VITAMIN B_{12} CONCENTRATIONS OF NORMALS AND OF PATIENTS WITH PERNICIOUS ANAEMIA IN RELAPSE AND WITH VARIOUS DISEASES OTHER THAN MEGALOBLASTIC ANAEMIA

Group	Age	No. of Subjects	Total Vitamin B_{12} Concentration ($\mu\mu$g. per ml.)			Combined Vitamin B_{12} Concentration ($\mu\mu$g. per ml.)		
			Range	Mean	S.E. of Mean	Range	Mean	S.E. of Mean
1. (a) Normal subjects	15–40	65	100–720	358	±16·1	100–620	329	±14·6
(b) Healthy subjects attending hospital with minor complaints	51–70	27	110–620	322	±28·9	110–540	295	±27·7
(c) Healthy subjects attending hospital with minor complaints	71–93	31	60–600	259	±31·6	30–600	234	±29·1
2. Patients with miscellaneous diseases ..	5–76	63	60–680	316	±20·2	40–680	298	±19·9
3. Patients with pernicious anaemia in relapse	28–74	43	<16–105*	43†	±3·3	<16–105*	43†	±3·3

* The range in this group is based on the observations made on 87 sera.
† The mean is based on 63 observations, because the values of 24 sera were too low to be measured. The true mean is therefore less than the recorded figure.

TABLE II

TOTAL AND COMBINED SERUM VITAMIN B_{12} CONCENTRATIONS OF PATIENTS (GROUP 2 OF TABLE I) WITH DISEASES OTHER THAN MEGALOBLASTIC ANAEMIA

Disease	No. of Patients	No. of Observations	Age	Total Vitamin B_{12} Concentration ($\mu\mu$g. per ml.)		Combined Vitamin B_{12} Concentration ($\mu\mu$g. per ml.)	
				Range	Mean	Range	Mean
Haemolytic anaemia ..	6	7	45–74	140–600	354	140–600	315
Polycythaemia vera ..	6	6	39–53	90–420	228	90–420	228
Acute leukaemia ..	6	6	6–35	180–400	336	175–400	306
Chronic leukaemia and generalized reticulosarcoma	6	6	33–74	150–400	356	150–400	346
Multiple myeloma ..	2	3	54–64	80–200	120	40–160	97
Iron-deficiency anaemia	7	8	32–73	130–680	325	130–680	325
Haemorrhagic diseases ..	3	5	39–54	130–440	234	90–440	226
Aplastic anaemia ..	1	1	18	170	—	170	—
Acute infections	4	4	24–35	240–470	365	240–470	352
Miscellaneous conditions‡	22	22	41–73	100–680	360	50–680	340

‡ This group included patients suffering from cardiac failure, various neurological conditions, including alcoholic neuritis, and various types of carcinoma. One patient was suffering from severe scurvy.

In Fig. 1 the vitamin B_{12} concentrations of the sera of these patients has been plotted against the red cell counts at the time the sera were obtained. In each case the concentration was below the normal range, and tended to be lower in the more anaemic patients. It was our impression that the lowest levels were found in those

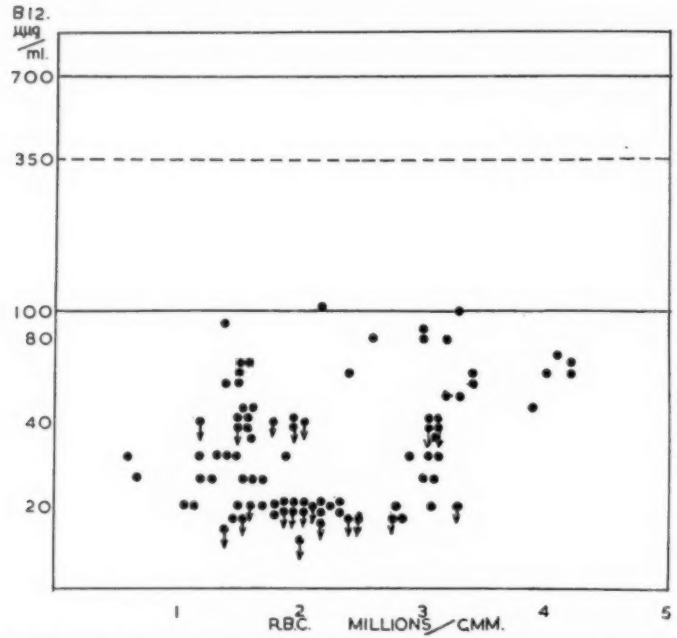


FIG. 1.—The serum vitamin B_{12} concentrations found in 43 patients with pernicious anaemia in relapse are plotted against the patient's red cell count at the time the serum was obtained. The limits of the normal range are indicated by the horizontal unbroken lines; the mean normal serum concentration is indicated by the broken line.

patients in whom the disease was progressing rapidly. Where the blood count was static rather higher levels were found, particularly when patients had red cell counts of 2.5 m. per c.mm. or more.

Almost half the samples of sera were obtained from patients receiving a normal diet; the other samples were taken after the patients had been deliberately placed on a diet of low vitamin B_{12} content. No significant difference in the serum concentrations was found between these two groups and no spontaneous remissions occurred during the periods of observation.

Subjects of Same Age and Economic Groups as Patients with Pernicious Anaemia.—The normal range given in Table I was based on observations made on healthy doctors, technicians, and nurses. The patients with pernicious anaemia on whom studies were made were older, poorer, and less well cared for, and therefore it seemed desirable to measure the vitamin B_{12} concentrations of the sera of non-anaemic subjects of similar age and living under comparable conditions. For this purpose sera were obtained from patients who were attending the out-patient department with minor medical or surgical conditions or were in hospital fully convalescent from minor ailments. The results are shown in Table I, Groups 1b and 1c.

TABLE III

INITIAL RED CELL COUNTS, SERUM VITAMIN B_{12} CONCENTRATIONS, ASSOCIATED CONDITIONS, AND

TABLE III

INITIAL RED CELL COUNTS, SERUM VITAMIN B_{12} CONCENTRATIONS, ASSOCIATED CONDITIONS AND RESPONSES TO THERAPY IN PATIENTS WITH MEGALOBLASTIC ANAEMIA OTHER THAN PERNICIOUS ANAEMIA

Case No.	Sex	Age	Associated Condition	Vitamin B_{12} Concentration of Serum ($\mu\mu$g. per ml.)	Therapy	Red Cell Count (m. per c.mm.)		Reticulocyte Response			Bone Marrow Findings		Remarks
						Initially	15 Days after Therapy	Initial %	% at Peak of Response	Peak Day	Initially	4 Days after Therapy	
1	F.	57	Ileo-transverse colostomy 3 yrs. previously	<20	20 μg. B_{12} intramuscularly	1·4	2·5	1·0	48	7th	Meg.*	Norm.†	Histamine-fast achlorhydria. Fat absorption: 87% on diet containing 50 g. fat (6-day balance)
2	F.	33	Tabes mesenterica and entero-anastomosis 6 yrs. previously	60 (2 observations	20 μg. B_{12} intramuscularly	2·4	3·2	5·0	22	8th	Meg.	Norm.	Free HCl in gastric juice after histamine: 44% of dried weight of faeces was fat
3	M.	51	Total gastrectomy. 2 yrs. previously. Ilio-iliostomy 18 months previously	20–40 (6 observations)	40 μg. B_{12} intramuscularly	1·9	2·8	<0·1	10	6th	Meg.	Norm.	Severe fatty diarrhoea 2 mths. Fat absorption: 30% on diet containing 50 g. fat (6-day balance)
4	F.	56	Total gastrectomy Oesophago-jejunostomy 2 yrs. previously	50 (2 observations)	Spontaneous remission	1·4	—	2·0	27	—	Meg.	Norm.	Spontaneous remission followed enrichment of diet with crude casein. The remission was associated with a rise in the serum B_{12} concentration to within the normal range. Fat absorption: 80% on diet containing 50 g. fat (6-day balance)
5	F.	34	Pregnancy	350–370 (3 observations)	40 μg. B_{12} intramuscularly	2·3	1·8	1·0	4	7th	Meg.	Meg.	Free HCl in gastric juice 2 yrs. previously when admitted with similar condition. Negligible response to vitamin B_{12}.
				230–250 (2 observations)	60 μg. folinic acid intramuscularly	2·2	2·3	5·0	11	5th	Meg.	Meg.	Good subjective response, but negligible haematological response to a very small dose of folinic acid
				320–350 (3 observations)	6 mg. folinic acid intramuscularly	2·3	3·2	8·0	21	6th	Meg.	Norm.	Good response to single injection of 6 mg. folinic acid
6	F.	58	? Idiopathic steatorrhoea	600	120 μg. B_{12} intramuscularly	1·4	1·1	0·4	12	9th	Meg.	Meg.	Initial R.B.C. count 0·4 m. per c.mm. Transfused before treatment. Free HCl in gastric juice. Fat absorption: 80% on diet containing 50 g. fat (6-day balance)
					20 mg. folic acid daily	1·1	2·7	4·0	47	6th	Meg.	Norm.	
7	M.	53	Idiopathic steatorrhoea	25	60 μg. B_{12} intramuscularly	0·8	—	0·5	—	—	Meg.	—	Free HCl in gastric juice. Diagnosed as suffering from idiopathic steatorrhoea elsewhere on the history and on the findings of a fat balance. Responded to treatment with vitamin B_{12}

* Megaloblastic haemopoiesis. † Normoblastic haemopoiesis.

The mean serum level of the patients aged 51–70 years (Table I, Group 1b) was lower than that of the healthy subjects aged 15 to 40 years, and was similar to that of the patients suffering from serious conditions (Table I, Group 2). The apparent difference was, however, not statistically significant. The mean total and combined serum vitamin B_{12} concentrations of the patients aged between 71 and 93 years was, however, significantly lower (Table I, Group 1c) than that of the normal group ($P < 0.001$). Many of them had disabilities common to their age group, but none was seriously ill or anaemic.

Patients with Megaloblastic other than Pernicious Anaemia.—Seven patients were studied. The initial red cell counts, serum vitamin B_{12} concentrations, associated conditions, and responses to therapy are shown in Table III. The serum vitamin B_{12} concentrations of five patients (Cases 1 to 4 and Case 7) were as low as those found in patients with pernicious anaemia; three of these patients (Cases 1 to 3) responded excellently to intramuscular injections of vitamin B_{12}. One patient (Case 4) developed a spontaneous remission before treatment which was associated with an increase of the serum vitamin B_{12} concentration to within the normal range. One patient (Case 7), treated at another hospital, also responded to treatment with vitamin B_{12} by injection.

Two patients, one with megaloblastic anaemia of pregnancy and one with a megaloblastic anaemia of doubtful aetiology, possibly idiopathic steatorrhoea, had normal serum vitamin B_{12} concentrations before treatment. The first patient (Case 5) failed to show a significant red cell or reticulocyte response to an injection of 40 μg. of crystalline vitamin B_{12}, but responded well to a single injection of 6 mg. of folinic acid. The other (Case 6) failed to respond to a single injection of 120 μg. vitamin B_{12}, but responded maximally to treatment with folic acid.

Diurnal and Day-to-day Observations in Normals and Patients with Pernicious and Other Anaemias.—Serum was obtained at frequent intervals during 48 hours from two normal subjects. The values found in one subject ranged from 290 to 350 $\mu\mu$g. per ml., and in the other from 105 to 130 $\mu\mu$g. per ml. (Table IV). Although the diet was not restricted, no relation was noted between the vitamin B_{12} concentration and the taking of food. In Table IV are also given the results of similar assays carried out over an eight-hour period on four patients with iron-deficiency anaemia, on one patient with aplastic anaemia, and on one patient with pernicious anaemia in relapse. These patients were fasting while the studies were made.

Serum was also taken at frequent intervals from two normal subjects for three weeks. The concentrations found are shown graphically in Fig. 2. The levels in one patient ranged from 100 to 210 $\mu\mu$g. per ml., and in the other from 110 to 400 $\mu\mu$g. per ml. In Fig. 2 are also shown the vitamin B_{12} concentrations of the serum of a patient with pernicious anaemia in relapse studied over a similar period. The values found ranged between 16 and 90 $\mu\mu$g. per ml. Serial assays were also carried out on the serum of a patient with iron-deficiency anaemia during the intense regeneration produced by oral iron therapy. The vitamin B_{12} concentration of the serum did not alter significantly during 30 days.

Uncombined Vitamin B_{12} in Serum.—Uncombined (free) material with growth activity for the Euglena similar to vitamin B_{12} was present in the sera of 54 subjects of the groups included in Table I in concentrations of from 40 to 160 $\mu\mu$g. per ml. (mean 81 $\mu\mu$g. per ml.). In the sera of all the patients with pernicious anaemia, and

TABLE IV

DIURNAL OBSERVATIONS IN TOTAL SERUM VITAMIN B_{12} CONCENTRATION OF TWO NORMALS AND SIX ANAEMIC PATIENTS

Clinical Condition	Vitamin B_{12} Serum Concentration ($\mu\mu$g. per ml.)					
	0	4 hrs.	8 hrs.	12 hrs.	24 hrs.	48 hrs.
Normal	345	290	320	320	305	290
,, ...	120	115	130	120	130	105
Iron-deficiency anaemia	170	230	210	—	—	—
,, ,, ,, ...	680	640	720	—	—	—
,, ,, ,, ...	150	120	120	—	—	—
,, ,, ,, ...	215	225	265	—	—	—
Aplastic anaemia	170	170	255	—	—	—
Pernicious anaemia in relapse	45	45	40	—	—	—

of the remaining 132 subjects included in this Table, uncombined material was present in amounts of less than 40 $\mu\mu$g. per ml.

Observations made on 15 sera were excluded from Table I because the serum vitamin B_{12} concentrations were far higher than those found in other sera. These high values, which ranged from 1,000 to more than 4,000 $\mu\mu$g. per ml., were due to the presence of large amounts of uncombined material, the combined vitamin B_{12} concentrations not being raised above the usual levels found in sera of the respective groups. These high free concentrations have been found in the sera of normal subjects and of patients with pernicious anaemia in relapse. Samples of sera taken 24 hours before or after the specimen containing the high free concentration have usually contained no free material. In one normal subject, nevertheless, large amounts were present in four specimens taken during a 12-hour period. In one patient with pernicious anaemia specimens of serum containing no free material were obtained three hours before and three hours after a sample had been taken which contained a very high concentration of this substance.

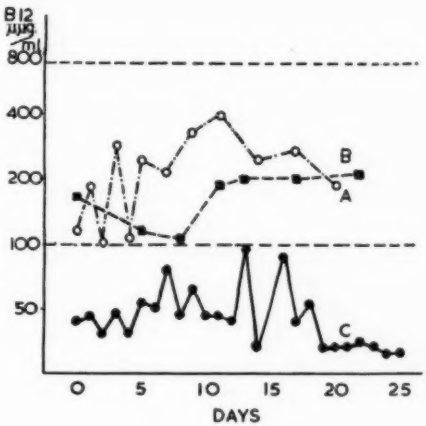


FIG. 2.—The variation in the total vitamin B_{12} concentration of the serum of two normal subjects and of one patient with pernicious anaemia in relapse during 25 days. The limits of the normal range are indicated by the horizontal broken lines. Curves A and B represent the changes in the serum concentration of the normal subjects, Curve C the serum concentration of the patient with pernicious anaemia.

Vitamin B_{12} Concentration of Urine.—The amount excreted in the urine in 24 hours by six normal subjects is given in Table V. The total daily excretion ranged from 0.11 to 0.27 μg.

The daily urinary excretion by eight patients with pernicious anaemia in relapse is given in Table VI. The values found, with one exception, were much lower than those found in the normal subjects. In seven patients less than 0.05 μg. was excreted daily; in a specimen from one patient the excretion was within the normal range.

TABLE V

DAILY EXCRETION OF VITAMIN B_{12} IN URINE OF SIX NORMALS

Volume of Urine (ml.)	Vitamin B_{12} Concentration ($\mu\mu$g. per ml.)	Total Vitamin B_{12} Excretion (μg. in 24 hours)
1,740*	140	0·24
1,420	80	0·11
1,115	250	0·27
2,055	100	0·21
1,400	94	0·13
1,300*	116	0·16
1,900	85	0·16
1,350	115	0·16
1,570	100	0·16

* Three separate collections were made from the first subject and two collections from the fourth.

TABLE VI

DAILY EXCRETION OF VITAMIN B_{12} IN URINE OF EIGHT† PATIENTS WITH PERNICIOUS ANAEMIA IN RELAPSE

Volume of Urine (ml.)	Vitamin B_{12} Concentration ($\mu\mu$g. per ml.)	Total Vitamin B_{12} Excretion (μg. in 24 hours)
910	25	0·02
1,360	<40	<0·05
1,135	<20	<0·02
1,385	<20	<0·03
1,846	<20	<0·04
1,740	<16	<0·03
840	32	0·027
1,415	100	0·15

† In seven patients the amount of vitamin B_{12} excreted was much lower than that excreted by normal subjects. In the last patient the daily excretion was similar to the excretion in normal subjects.

Discussion

It is only very recently that the first results of the microbiological assay of vitamin B_{12} in body fluids have appeared in the literature. Ross (1950) in a short communication reported his preliminary results using *Euglena gracilis*, and Couch, Olcese, Witten, and Colby (1950), who used *L. leichmannii* as test organism, reported the results of assays made on the whole blood of 10 normal human subjects and of a number of animal species. The latter organism was also used by Chow, Lang, Davis,

Conley, and Ellicott (1950) for the assay of the vitamin B_{12} content of urine, but was found by them to be insufficiently sensitive to detect the vitamin in the urine of normal subjects. Girdwood (1951) has, however, reported the results of assays with this organism of the vitamin B_{12} content of the urine of normal subjects and patients suffering from megaloblastic anaemia. Though the values for normal subjects were similar to those reported here he did not demonstrate a clear-cut difference between the urinary excretion of the vitamin by normal subjects and patients with pernicious anaemia.

Euglena gracilis is a more sensitive test organism than the lactobacilli which have been used for vitamin B_{12} assay, and its use, as now reported, made it possible not only to demonstrate the presence of vitamin B_{12}-like material in serum and urine, but also to measure its range in health and disease. The concentrations of this material in the serum of normal subjects and of patients suffering from conditions other than megaloblastic anaemia were found in this study to be similar. The levels in the serum of two patients (Table III, Cases 5 and 6) suffering from megaloblastic anaemia refractory to treatment with vitamin B_{12} were not different from those found in normal subjects, but the serum concentrations of patients suffering from pernicious anaemia or other megaloblastic anaemias which responded to treatment with vitamin B_{12} were below the normal range.

The Specificity of the Euglena for Haemopoietically Active Vitamin B_{12}.—The observations summarized in the previous paragraph suggest that the material measured is actually vitamin B_{12}. The much smaller amount of vitamin B_{12}-like material found in the urine of patients with pernicious anaemia in relapse compared with normal subjects also supports this view. Nevertheless it is not certain that vitamin B_{12} is the only material in serum and urine which is capable of promoting the growth of the Euglena. As already mentioned, we excluded the observations made on 15 sera from the results given in Table I. These sera were excluded because their total vitamin B_{12} equivalent was far higher than that found in the other 186 sera examined. This high total concentration was due to the presence of considerable amounts of uncombined material having a vitamin B_{12}-like activity for the Euglena. Five of these sera were from patients with pernicious anaemia in relapse, but in spite of the high concentrations found, which would have been equivalent to as much as 6 μg. of vitamin B_{12} circulating in the blood stream, no haematological remission occurred. Yet the same patients subsequently responded well to parenteral or oral administration of the vitamin. Two explanations of this finding seem possible. First, material may be actually present in the blood stream of these patients and, although not haemopoietically active, have growth activity for the Euglena similar to vitamin B_{12}. If this is so, it may possibly be of cobalamin nature and enter the blood from either intestine or the tissues. Secondly, material may not be an actual constituent of the patient's blood, but may result from contamination of the blood during its collection and preparation for assay either from needle, syringe, or glassware previously in contact with vitamin B_{12}, or with material with similar activity for the Euglena. The method of assay is so sensitive that traces of vitamin B_{12} which might remain after thorough washing and sterilization of syringes and needles previously used for injecting vitamin B_{12} could conceivably affect results. Throughout the investigation every effort was made to ensure that the syringes and needles used for administration of vitamin B_{12} were kept apart from

those used for withdrawal of blood. Nevertheless, it is possible that in certain instances this arrangement may have broken down. The presence of free material was not confined to the 15 sera just referred to. As already mentioned, other sera contained free material in measurable though small amounts. The nature and source of this are also uncertain.

The total concentration of material having a vitamin B_{12}-like activity for the Euglena may not, therefore, in all cases be an accurate measure of the amount of haemopoietically active vitamin B_{12} present in the patient's blood stream. However, the concentration of *combined* vitamin B_{12}-like material seems to be closely correlated with the nutritional state of the body in respect of vitamin B_{12}. In all patients suffering from megaloblastic anaemias which responded to treatment with vitamin B_{12} the serum concentrations of the combined material were below the range of the concentration of this material in normal subjects. In no other conditions were such low concentrations found. Further, when vitamin B_{12} was given parenterally to patients with pernicious anaemia in relapse the serum concentrations of the combined material increased to within the normal range, and, as long as this concentration remained above the lower level of the normal range, haemopoiesis in the bone marrow was normoblastic. When the combined concentration fell below the limits of the normal, megaloblastic haemopoiesis reappeared.

The observation that the combined concentration reflects the body's needs for vitamin B_{12} is of interest in view of the demonstration by Ternberg and Eakin (1949) that crystalline vitamin B_{12} is bound by material present in gastric juice. This bound form of vitamin B_{12}, like the combined form of the material in serum, is not available to micro-organisms until freed from combination. Callender and Lajtha (1951) have also shown that, although crystalline vitamin B_{12} alone had no effect in maturing megaloblasts in tissue culture, the incubated mixture of vitamin B_{12} and normal gastric juice was effective in this respect. Normal serum, in which, presumably, the vitamin was in the combined form, was also effective. The true significance of the combination of vitamin B_{12} with components present in serum and gastric juice is still uncertain, as it is known that vitamin B_{12} combines with great facility with a wide variety of protein-like substances (Meyer, Eppstein, Bethell, and Hall, 1950; Beerstecher and Altgelt, 1951; Bird and Hoevet, 1951). Some of these combinations are not specifically active in patients with pernicious anaemia (Hall, Morgan, and Campbell, 1950).

Serum Vitamin B_{12} Concentrations and Megaloblastosis of the Bone Marrow.—The vitamin B_{12} concentrations found in the patients with pernicious anaemia, all of whom showed recognizable megaloblastic haemopoiesis on marrow biopsy, ranged from less than 16 to 105 $\mu\mu$g. per ml. No free material was present in these sera. The upper limit of the serum vitamin B_{12} concentration at which it is possible to recognize the presence of megaloblastic change in the marrow is probably between 80 and 100 $\mu\mu$g. per ml. These levels have been found in patients who showed only minimal signs of anaemia as judged by an examination of the peripheral blood. Serum concentrations of 60 to 100 $\mu\mu$g. per ml. have been found in single specimens of serum taken from three patients in the 71 to 93 age group not proved to have been suffering from a megaloblastic anaemia. Similarly in one patient with multiple myeloma and hyperglobulinaemia, total serum vitamin B_{12} concentrations of 80 and 100 $\mu\mu$g. per ml., 40 to 60 $\mu\mu$g. of which was combined material, were found on two occasions.

Although the patient was anaemic, haemopoiesis was normoblastic at the time of the first serum assay. Thus, serum concentrations below 80 $\mu\mu$g. per ml. may very occasionally not be associated with detectable clinical or haematological signs of deficiency. Whether such low values are due to technical errors in preparing specimens for assay, or to the error of the assay method itself, or to another cause, is uncertain.

Summary

The vitamin B_{12} concentrations of the serum and urine of normal subjects, of patients with megaloblastic anaemia, and of patients suffering from various other conditions have been assayed, using *Euglena gracilis* as the test organism.

The total concentrations in the sera from normal subjects ranged from 100 to 720 $\mu\mu$g. per ml. (mean 358 $\mu\mu$g.). The combined vitamin B_{12} concentration ranged from 100 to 620 $\mu\mu$g. per ml. (mean 329 $\mu\mu$g.). Similar values were found in sera from patients suffering from conditions other than megaloblastic anaemia.

The vitamin B_{12} concentrations of the sera of patients suffering from pernicious anaemia in relapse and of patients with certain other types of megaloblastic anaemia which responded to treatment with vitamin B_{12} were below the normal range. The amount of vitamin B_{12} excreted daily in the urine by these patients was much less than the amount excreted by normal subjects. Patients suffering from megaloblastic anaemia which was refractory to treatment with vitamin B_{12} were found to have normal serum vitamin B_{12} concentrations.

The material in sera which has a vitamin B_{12}-like activity for the growth of the Euglena was present in some sera in both an uncombined and combined form. Uncombined material was only found in 30% of sera, usually in small concentrations. Certain sera, however, contained large amounts of uncombined material. The significance of this uncombined material is discussed, and reasons are given for believing that the concentration of the combined material represents haemopoietically active vitamin B_{12}.

We wish to thank Dr. J. V. Dacie for advice and encouragement during this investigation; the physicians at Hammersmith Hospital for their help and co-operation; Dr. J. M. Chalmers, Dr. G. A. Matthews, and Dr. A. I. Ross for sera from patients under their care; Glaxo Laboratories Ltd. for supplying the vitamin B_{12} used in this study; Lederle Laboratories Division, Cyanamid Products Ltd., for the folinic acid used; Miss J. Davenport, Mrs. H. M. Stephen, Mr. L. H. Turnbull, and Mr. L. Wallett for technical assistance.

References

Beerstecher, E., and Altgelt, S. (1951). *J. biol. Chem.*, **189**, 31.
Bird, O. D., and Hoevet, B. (1951). Ibid., **190**, 181.
Callender, S. T. E., and Lajtha, L. G. (1951). Quoted by Dacie, J. V., and Witts, L. J., *Blood*, **6**, 586.
Chow, B. F., Lang, C. A., Davis, R., Conley, C. L., and Ellicott, C. E. (1950). *Bull. Johns Hopk. Hosp.*, **87**, 156.
Couch, J. R., Olcese, O., Witten, P. W., and Colby, R. W. (1950). *Amer. J. Physiol.*, **163**, 77.
Girdwood, R. H. (1951). *Edinb. med. J.*, **58**, 309.
Hall, B. E., Morgan, E. H., and Campbell, D. C. (1950). *Brit. med. J.*, **2**, 585, 960.
Hutner, S. H., Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L., and Jukes, T. H. (1949). *Proc. Soc. exp. Biol., N.Y.*, **70**, 118.
Meyer, C. E., Eppstein, S. H., Bethell, F. H., and Hall, B. E. (1950). *Fed. Proc.*, **9**, 205.
Ross, G. I. M. (1950). *Nature, Lond.*, **166**, 270.
—— (1952). To be published.
Ternberg, J. L., and Eakin, R. E. (1949). *J. Amer. chem. Soc.*, **71**, 3858.

J. clin. Path. (1952), **5**, 140.

STUDIES ON THE PATHOGENESIS OF THALASSAEMIA

BY

GIOVANNI ASTALDI AND PAOLO TOLENTINO

From the Departments of Internal Medicine, University of Pavia, and of Paediatrics, University of Genoa, Italy

(RECEIVED FOR PUBLICATION SEPTEMBER 27, 1951)

This short paper is a study of erythrocyte production and destruction in thalassaemia, undertaken so as to throw light on the pathogenesis of the disease. Thalassaemic bone marrow has been cultured *in vitro* (Astaldi, Tolentino, and Sacchetti, 1951a, b, c) thereby allowing the separate study of the intensity of differentiation and the proliferation of erythroblasts. By the culture method it is possible to visualize these activities more exactly than by static morphological criteria such as the myelogram, the maturation curve, the mitotic rate, and the karyokinetic histogram.

Method

Differentiation.—Differentiation was studied by keeping the surviving medullary cells in a Carrel flask containing a liquid medium consisting of plasma and Tyrode's solution (Astaldi and Tolentino, 1949). At the beginning of the experiment, and then at six to 12-hour intervals, a count was made in absolute numbers per c.mm. of the erythroblasts and reticulocytes (at different stages of maturation) surviving in the culture.

As under these conditions erythropoiesis quickly fades while differentiation continues, the total number of erythroblasts gradually decreases and may ultimately disappear. The first to diminish in numbers are the basophilic erythroblasts followed by the polychromatic and finally by the orthochromatic erythroblasts. The reticulocytes, too, disappear as they ripen to adult erythrocytes.

Proliferation.—Proliferation was studied by explanting fragments of bone marrow on to solid medium containing colchicine at an optimum concentration (Astaldi and Mauri, 1949, 1950). Owing to the "stathmokinetic" action of the colchicine, further development of the cells entering mitosis is inhibited at pre-metaphase, the result being that the number of mitoses in the culture (the "stathmokinetic rate") gradually increases with time. The rapidity of proliferation can thus be deduced from the increase in the number of mitoses reached in a given time.

Results

Proliferation.—The mitotic activity of the basophilic erythroblast was found in all the three forms of thalassaemia (major, minor, and minima) to be far greater than that of the basophilic erythroblasts of a normal subject. In thalassaemia major and minor the rate is constantly high; in thalassaemia minima it is more variable (Fig. 1). On the other hand, the proliferation activity of the polychromatic erythroblasts is no greater than normal in all three forms of thalassaemia. The orthochromatic erythroblasts do not divide.

Differentiation.—It is possible that in all three forms of thalassaemia there is some delay in the development of the polychromatic from the basophilic erythro-

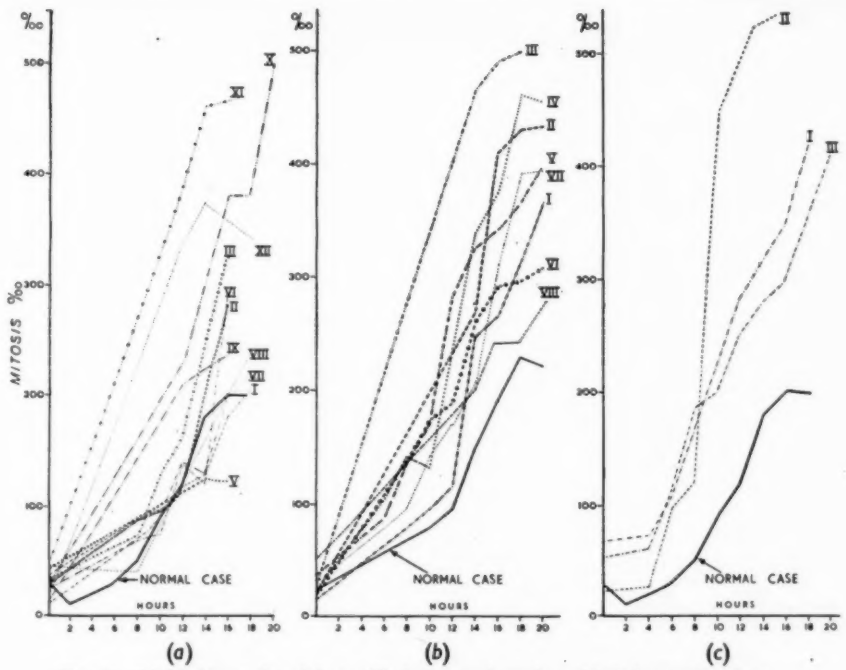


FIG. 1.—(*a*) Thalassaemia minima; (*b*) thalassaemia major; (*c*) thalassaemia minor.

blast. In the most serious cases of thalassaemia major the differentiation of orthochromatic erythroblasts into erythrocytes is retarded (delayed enucleation), this phenomenon being easier to observe because the erythroblasts do not proliferate at the orthochromatic stage (Fig. 2). Such a phenomenon is, however, not exclusively associated with thalassaemia ; it has been observed in other secondary erythroblastic anaemias associated with hyperhaemolysis.

The medullary erythroblastosis which is found in all three forms of thalassaemia can be explained by an increased formation of erythroblasts from the histoid matrix accompanied by increased proliferation of the basophilic erythroblasts. In the worst cases this may be associated with a slowing down in the differentiation of the orthochromatic erythroblasts.

" Crossed " Plasma Experiments.—Experiments have been carried out using a " crossed " plasma technique, thalassaemic marrow being cultured in normal plasma and vice versa. It has been observed that thalassaemic basophilic erythroblasts proliferate in normal plasma and in thalassaemic plasma at the same degree of intensity (Fig. 3) ; also that the proliferation of normal erythroblasts does not increase in thalassaemic plasma (Fig. 3), and that the differentiation of thalassaemic erythroblasts follows the same course in thalassaemic plasma as it does in normal plasma. Similarly, the differentiation of normal erythroblasts is not affected by

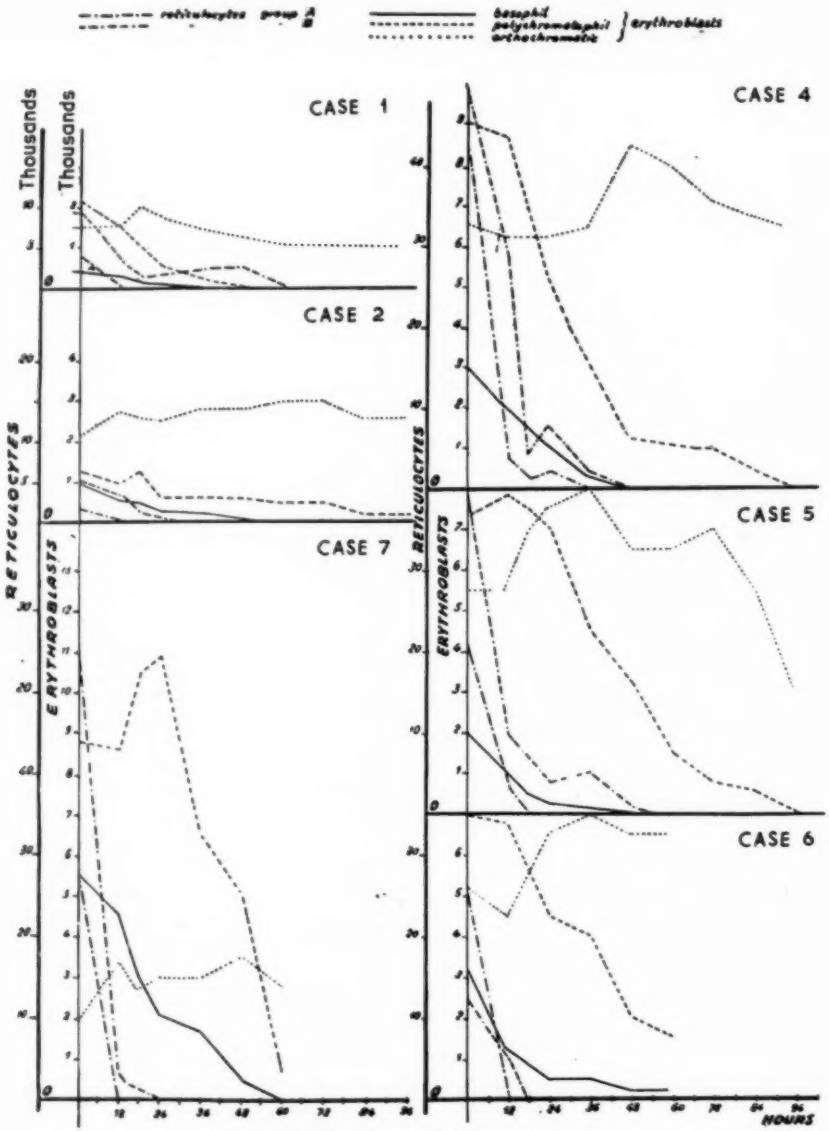


FIG. 2.—Differentiation of thalassaemia major.

thalassaemic plasma. It can thus be inferred that the abnormal proliferation and differentiation of the erythroblasts in thalassaemia are inherent characteristics, not affected by the type of plasma in which the cells are cultured.

Discussion

Compensatory Erythropoiesis in Thalassaemia.—The considerable increase in the rate of erythropoiesis in thalassaemia is matched by an increase in the rate of erythrocyte destruction so that a proportional relationship exists between the two phenomena. Both are less intense in thalassaemia minima than in the minor and major forms. Thalassaemia minima may in fact be accompanied by polycythaemia, in spite of the existence of increased haemolysis which, though not so marked as in the major and minor forms, may bring about erythrocyte destruction at twice the normal rate (Astaldi, Tolentino, Sacchetti, and Nonato, 1951).

The erythroblastosis of the bone marrow is thus considered to be a result of the increased haemolysis rather than a primary ana-dysplastic erythroblastosis. In thalassaemia minima, where the destruction of erythrocytes is not very excessive, compensation is complete, or there may be over-compensation and polycythaemia. In the minor and major forms, on the other hand, the rate of destruction is so great that the losses cannot be made good.

Mechanism of Haemolysis.—Of the three mechanisms by which erythrocytes may be removed from the circulation, fragmentation, lysis, and phagocytosis, the first seems to be the most important in thalassaemia. To it must be attributed the bizarre forms of erythrocytes and the smallest (2–3 μ) microcytes. This phenomenon does not seem to be due to an increased erythrocyte mechanical fragility, for

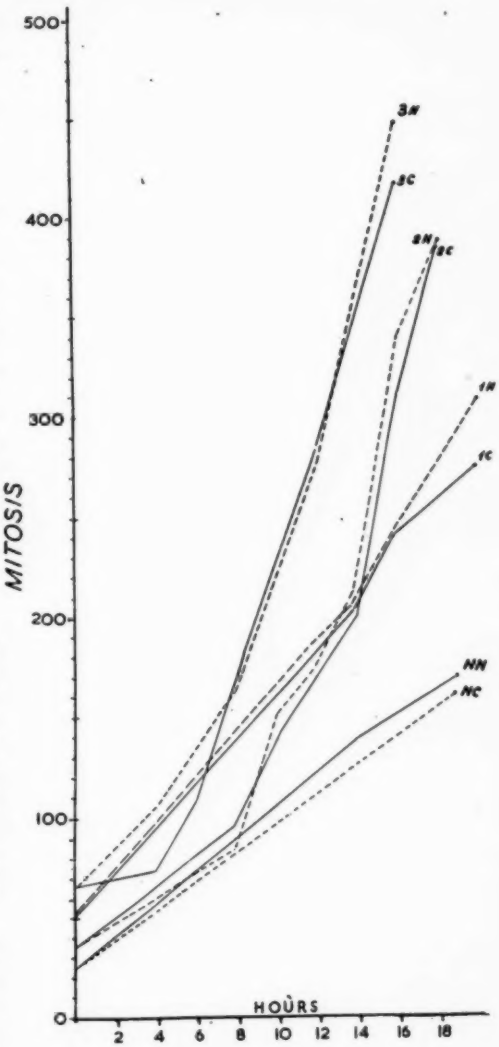


FIG. 3.—Shows "crossed plasma" proliferation. 1C, 2C, 3C = thalassaemia erythroblasts in autoplasma. 1N, 2N, 3N = thalassaemia erythroblasts in normal plasma. NN = normal erythroblasts in autoplasma. NC = normal erythroblasts in thalassaemia plasma.

tests *in vitro* have shown a higher resistance to trauma (Tolentino, 1950, 1951). The process of spontaneous fragmentation seems rather to be due to intrinsic causes, and may even be seen to be taking place in young forms (reticulocytes).

The modifications produced as a result of splenectomy are not such as support the existence in thalassaemia of a hypersplenic condition. On the contrary, the evidence is in favour of hyposplenism. Similarly, the evidence is against the hypothesis that an abnormality in the plasma determines the increased haemolysis. In fact, the results all point to the conclusion that this increased rate of blood destruction is due to the abnormality of the erythron itself, this notwithstanding the fact that the medullary erythroblastosis is not the morphological expression of such an abnormality but only the consequence of the increased peripheral destruction.

Summary

The pathogenesis of thalassaemia can thus be summarized as follows. The disease is due to a gene mutation of variable expressivity (Gatto, 1947 ; Silvestroni, 1947) which affects the erythron and causes the formation of morphologically and biologically abnormal erythrocytes. The biological anomaly determines a shorter average life span, resulting in a permanent increase in the rate of haemolysis. A chronic stimulation of the bone marrow with secondary hyperplasia results. The hyperplasia depends on an increased proliferation of basophilic erythroblasts and probably also on a direct differentiation of the histoid matrix.

In thalassaemia minima (the carrier condition) the increase in erythropoiesis makes up for the increased rate of haemolysis and anaemia is avoided. The mating of two thalassaemia minima carriers can result in the birth of homozygous children and these will be afflicted with thalassaemia major. The summation of the effects of two mutant genes prevents the possibility of erythropoiesis balancing haemolysis, for the life span of the erythrocyte is then shorter than in the minima form. The compensatory erythropoiesis is inadequate, even after maximal proliferation of the basophilic erythroblasts and an increase in histoid erythropoiesis to the extent of filling up all the medullary cavities with active erythroblastic tissue, enlarging the diplöe, and invading areas and organs which have as a rule no erythropoietic activity. In such cases the anaemia becomes progressively worse, and periods of bone marrow exhaustion may result in a slowing down of erythroblastic differentiation. The enucleation of orthochromatic erythroblasts is particularly affected and marked erythroblastaemia is evidence of this.

In thalassaemia minor, which is, like thalassaemia minima, a heterozygous condition, the picture is similar to that of thalassaemia major. It is less severe, however, and is not incompatible with life.

References

Astaldi, G., and Mauri, C. (1949). *Haematologica*, **33**, 583.
—— —— (1950). *Sang*, **21**, 378.
—— and Tolentino, P. (1949). *Journal of Clinical Pathology*, **2**, 217.
—— —— and Sacchetti, C. (1951a.) *Rev. Hémat.*, **6**, 157.
—— —— —— (1951b). *Helv. paediat. Acta*, **6**, 50.
—— —— —— (1951c). *La Talassemia.* (Bibl. Haemat.) Tipografia del Libro. Pavia.
—— —— —— and Nonato, M. G. (1951). *Acta haemat., Basel*, **5**, 270.
Gatto, I. (1947). *Riv. Pediatr. Sicil.*, **2**, 20.
Silvestroni, E. (1947). *Policlinico, Sez. prat.*, **54**, 1147.
Tolentino, P. (1950). *Boll. Soc. ital. Biol. sper.*, **26**, 251.
—— (1951). *Nature, Lond.*, **167**, 905.

J. clin. Path. (1952), **5**, 145.

THE BONE MARROW IN THE ANAEMIA OF PREGNANCY

BY

JEAN M. SCOTT AND A. D. T. GOVAN

From the Glasgow Royal Maternity and Women's Hospital

(RECEIVED FOR PUBLICATION AUGUST 31, 1951)

We have already shown that the incidence of anaemia among pregnant women is high in Glasgow, namely 20% (Scott and Govan, 1949). The majority have an iron deficiency anaemia, but at least 4.3% of those patients with a microcytic hypochromic blood picture do not respond completely to iron therapy and only show further improvement once liver in some form is given.

It is now well recognized that the megaloblastic anaemias of pregnancy may present a completely atypical blood picture (Stevenson, 1938 ; Abramson, 1938 ; Segerdahl, 1941 ; Davidson, Davis, and Innes, 1942b ; Callender, 1944 ; Wolff and Limarzi, 1946), and to-day haematologists tend more and more to diagnose this condition from the bone marrow picture. Thus within the last 10 years studies of larger numbers of cases have appeared, and a condition which at one time was considered rare (Beckman (1921) reported six in 60,000 normal deliveries) has been given greater prominence. Indeed, opinions would appear to have veered in the opposite direction, for in some of Segerdahl's (1941) cases a diagnosis of pernicious anaemia of pregnancy was made when megaloblasts were as low as 0.6%. It is doubtful whether this figure alone can be regarded as pathognomonic. Small percentages of cells resembling megaloblasts have been reported in the marrows of normal pregnant women (Daniachij, 1936 ; Russo, 1937), although the majority of authors do not hold this opinion (Hansen, 1938 ; Forssell, 1939 ; Markoff, 1939 ; Callender, 1946).

The problem of the megaloblast is a difficult one. Differentiation of the megaloblastic marrow may not be easy, and Mallarmé (1948) recognized intermediary forms in his " incipient " cases of pernicious anaemia, while Leitner (1948) has suggested that there may even be a " hypochromic prepernicious " stage. In pregnancy differentiation is even more difficult because the marrows are dimorphic and the percentage of the megaloblasts may not be high. In this hospital an initial sternal puncture is a routine procedure on all cases of anaemia with haemoglobin readings less than 8 g. %. Though the majority of marrows examined were normoblastic and a few megaloblastic, there were others containing intermediary forms which were very difficult to classify.

This paper is an attempt to relate the marrow morphology to the type of anaemia and to determine the essential features of each variety. It is, therefore, necessary to establish certain diagnostic criteria entailing a description of some of the abnormal precursors in the red cell series. Normoblasts, megaloblasts, plasma

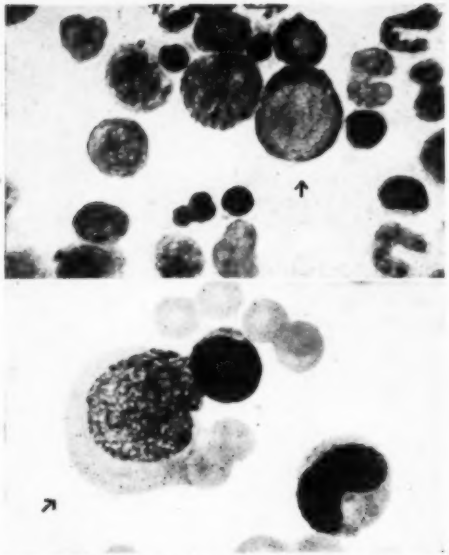


FIG. 1.—A, film showing a type b intermediate cell (× 1,200). B, film showing a type c smear cell (× 1,300).

cells, and reticulum are very fully described in the standard textbooks of haematology, but there are three abnormal cells which occur in the anaemias of pregnancy and require description.

Abnormal Cells in Anaemias of Pregnancy

Type a : Macronormoblast. — This has been described already (Whitby and Britton, 1946), but, as we have found it in small numbers in marrows other than those essentially macronormoblastic, we would state that it was larger than normal with a larger nucleus and a chromatin network more open than one would expect in comparison with the degree of haemoglobinization of the cytoplasm.

Type b : Intermediate Cell.—In many of the marrows an early cell was found which could not be placed in either the megaloblast or normoblast series, nor did it seem correct to call it an "early macronormoblast." About 20 μ in diameter, it had a large, pale pink, almost homogeneous nucleus whose chromatin network was never very distinct. Occasionally small condensations of the chromatin could be seen, but these were unusual. The cytoplasm was never a deep blue, but all other shades could be seen even to a clear sky-blue, when it resembled rather closely the typical megaloblast. Nucleoli could not usually be demonstrated by ordinary Romanowski stains.

Type c : Smear Cell.—This was a very immature cell, which, owing to the tendency to disintegrate and lose its cytoplasm when the marrow smears were made, was called a smear cell. Usually the nucleus was seen without any surrounding cytoplasm, but careful searching of the fields revealed that where the cytoplasm was attached it was clear blue. The nuclei appeared to be structurally similar to those of the promegaloblast. Vital staining brought this out more clearly and stained the nucleoli a clear sky-blue. From their appearance and relation to other cells these seemed to be red cell precursors, but this was not definite, and possibly they were similar to the cells described by Rohr (1940) as "large proliferated reticulum cells" and by Callender (1944) as "haemocytoblasts." We have included them with the plasma cells and reticulum cells in our differential counts.

Material and Methods

Forty-six patients were investigated. All had attended the antenatal clinic and were admitted to hospital for investigation. Sternal puncture was performed and smears made according to the method of Davidson, Davis, and Innes (1942b). The films were stained with May-Grunwald and cresyl blue and a differential count on 400 cells made in each case. Haemoglobin levels were estimated with a photoelectric colorimeter.

In the statistical analysis the standard error was calculated for each group and for the figures noted in Table I. In comparing the percentages Fisher's t test was applied, and the value of t checked by means of the angular transformation. (Only the significant points of the statistical analysis are mentioned here.)

Results

Of the 46 patients examined, 39 had a microcytic hypochromic blood picture. The remaining seven had macrocytic anaemias. Examination of the bone marrows showed that these cases could be divided into four groups.

Group 1.—This consisted of 11 patients with an average haemoglobin of 7.85 g.% and a red cell count of 3.63 m./c.mm. Haemopoiesis was normoblastic in every case and the cells of this series numbered 15.39%, the majority being polychromatic normoblasts. Macronormoblasts were rare and mitosis infrequent. Plasma cells and reticulum cells were not increased. Marrow reticulocyte counts were made, but in no case was the percentage higher than 1.0. All these patients were given iron, and they showed a very satisfactory response.

Group 2.—The second group of 17 patients included four who had already been treated with oral iron and had shown no improvement. Though their results are given separately in the table, they show such a marked similarity to the remaining 13 patients that we considered they should be included. The average haemoglobin was 5.2 g. % and the red cell count 3.16 m./c.mm. Both these readings are significantly lower than those in Group 1, but the drop in haemoglobin is the more striking. When the marrows were examined the striking feature was the marked degree of hyperplasia of the red cell series. Haemopoiesis was essentially normoblastic, but there was a significantly larger number of macronormoblasts. The normoblast count (including the macronormoblasts) was increased to 29.22%, and though this in itself was significant an analysis of the cells at each stage of maturation showed that the increase was most marked in the polychromatic normoblasts and less so in the basophilic cells. The early pronormoblasts and late orthochromatic normoblasts showed no significant change. Mitoses had increased to 1.4%, and the marrow reticulocyte count of 2.7% was also significantly increased. Plasma and reticulum cells showed no significant change. In almost every smear examined a few type b abnormal early cells were seen, yet every patient responded to iron, and the four patients who failed to improve on oral iron recovered completely on intravenous iron.

Group 3.—Seven patients formed this group and all had very severe anaemia. The average haemoglobin was 4.43 g.% and the red cell count 3.27 m./c.mm. The haemoglobin reading was significantly lower than that of Group 1 anaemias, but, due to wider variations in the red cell counts of the patients within this group (S.E. $\pm$0.42), the drop in the mean red cell count was not significant. The blood picture was very similar to that of Group 2 anaemias. Indeed, it was practically impossible to recognize these anaemias from their peripheral blood pictures, though occasionally a more marked anisocytosis was suggestive of the third group. In the bone marrow, however, distinguishing characteristics were apparent. Although haemopoiesis was still essentially normoblastic, typical megaloblasts were present to the extent of 2.4%. The total normoblast count was lower than in Group 2,

TABLE I

BONE MARROW FINDINGS IN FOUR GROUPS OF CASES OF ANAEMIA OF PREGNANCY

Group	No. of Cases	Hb (g. %)	R.B.C. (m./mm.³)	Reticulocytes (%)	Normoblasts				Megaloblasts				Macro-normoblasts			Abnormal Early Erythroblasts (%)	Normoblast Total (%)	Megaloblast Total (%)	Mitosis	
					Pronormoblast (%)	Basophilic (%)	Polychromatic (%)	Orthochromatic (%)	Promegaloblast (%)	Basophilic (%)	Polychromatic (%)	Orthochromatic (%)	Basophilic (%)	Polychromatic (%)	Orthochromatic (%)				Red Cell Series	White Cell Series
1	11	7·85 (0·27)*	3·63 (0·12)	—	0·52 (0·13)	1·52 (0·34)	7·44 (1·13)	5·22 (1·16)	—	—	—	—	0·05 (0·05)	0·49 (0·8)	0·15 (0·11)	—	15·39 (1·34)	—	0·19 (0·069)	0·14 (0·069)
2	17	5·22) (0·24)	3·16 (0·12)	2·72 (0·38)	0·90 (0·29)	3·46 (0·48)	13·63 (1·0)	6·69 (0·78)	—	—	—	—	0·67 (0·11)	2·52 (0·43)	1·35 (0·46)	0·9	29·22 (1·29)	—	1·43 (0·15)	0·2 (0·07)
3	7	4·43 (0·49)	3·27 (0·42)	2·57 (1·1)	0·50 (0·23)	2·26 (0·45)	5·46 (0·65)	5·71 (1·33)	0·1	0·6	0·9	0·8	0·89 (0·3)	3·40 (0·33)	2·18 (0·58)	0·6	20·40 (2·11)	2·44 (0·48)	0·75 (0·13)	0·21 (0·4)
4	6	5·41 (0·64)	2·13 (0·24)	0·67 (0·44)	1·08 (0·17)	2·13 (0·46)	2·04 (1·06)	2·17 (0·78)	1·42	1·04	1·29	0·79	1·04 (0·40)	1·79 (0·38)	1·38 (0·15)	1·0	11·63 (2·6)	4·54 (1·14)	0·54 (0·15)	0·21 (0·16)
	1	7·29	3·19	3·0	1·5	2·25	5·5	11·0	0·25	1·0	0·75	1·0	2·25	7·25	6·0	0·5	37·25	3·0	1·8	2·0
Post-haemorrhagic	4	4·8	2·03	3·8	0·43	1·3	7·5	9·8	—	—	—	—	0·9	1·9	1·6	—	23·4	—		

* Figures in brackets represent the standard error.

but it was significantly increased compared with Group 1 anaemias. Analysis of the individual cell-types showed a significant increase in the number of macronormoblasts, most marked at the basophilic stage and progressively less as development proceeded through the polychromatic to the orthochromatic stage. A slight decrease occurred in the ordinary normoblast series at every stage of development, but this was not statistically significant.

Despite the fact that these megaloblastic marrows did not give the impression of being specially active, nevertheless the number of cells in mitosis had increased to 0.75%, and the marrow reticulocyte count of 2.57% was also significantly raised. There was also an insignificant increase in the plasma and reticulum cells.

The patients of this group showed only a partial response to iron therapy and ultimately proved resistant even to intravenous iron. A change to liver therapy produced an immediate improvement with the appearance of a reticulocytosis in the peripheral blood.

Group 4.—This group consisted of seven patients with a macrocytic blood picture. The findings in six were fairly uniform. The seventh will be considered separately. Of the six patients, five were seen antenatally and one in the puerperium.

The average haemoglobin of these six patients was 5.41 g. % and the red count 2.13 m./c.mm.; i.e., compared with the Group 1 anaemias there was a significant drop in both haemoglobin and red cell levels, and, as would be expected, the fall in the red cells was the more striking.

The marrow was dimorphic in every case. Megaloblasts averaged 4.54% and normoblasts 11.63%. Although this was the lowest percentage of normoblasts of any group, the diminution was not statistically significant when compared with Group 1 anaemias. This was surprising, but detailed analysis of the normoblastic series showed that there was a marked diminution in the orthochromatic normoblasts and this was of definite statistical significance. The greater proportion of the normoblast series was composed of earlier forms of normoblasts and of macronormoblasts, a feature not seen in Group 1.

Reticulo-endothelial cells showed a very significant increase in the marrows of the macrocytic anaemias. This was mainly due to the presence of large immature smear cells, though the plasma cells themselves averaged as much as 3.2%. There was no increase in the marrow reticulocytes, but mitotic figures in the red cell series were more frequent and this was significant.

All six patients were given liver principle and showed a satisfactory response to treatment.

The remaining member of the group had a haemoglobin of 7.29 g. % and a red cell count of 3.19 million. Though the red cells were macrocytic the patient had also a marked hypochromia. The marrow contained 3% megaloblasts and 37.25% normoblasts, of which 15.5% were macronormoblasts. The marrow was very cellular and hyperplasia of the red cell series obvious. As can be seen from Table I, there were a considerably greater number of mature normoblasts compared with the other cases of macrocytic anaemia. In addition mitotic figures were frequent, 1.8%, and marrow reticulocytes, 3%. Plasma and reticulum cells were not exceptionally high. All these findings tended to suggest that the marrow hyperplasia in this case was producing more mature forms, and we decided to withhold liver

and review the case. Spontaneous recovery occurred, but the rate of improvement was slower than other cases treated with iron and liver.

Discussion

There is still considerable disagreement on the subject of "the bone marrow of pregnancy." Most authors admit that there is a slight tendency to moderate hyperplasia with the appearance of macronormoblasts, and report a shift to the left of the granular series as the pregnancy advances, but they have not found these results significant in comparison with non-pregnant women (Forssell, 1939; Pitts and Packham, 1939; Callender, 1946). Others, like Daniachij (1936), Hansen (1938), and Markoff (1939), disagree with this conclusion and feel justified in speaking of a bone marrow of pregnancy. Daniachij also reported the presence of megaloblasts in his cases. Callender (1946) and Montoya Gómez (1947) disagreed with Daniachij, and we too have never found any megaloblasts in the normal cases we have examined. Anaemia in pregnancy does not usually become apparent till the third trimester, and none of our cases was seen earlier than this. In order to establish criteria we have selected a few of the reports on the marrows of normal pregnant women seen in the last three months of pregnancy (Table II). Pitts and Packham's (1939)

TABLE II

BONE MARROW FINDINGS IN NORMAL PREGNANT WOMEN

Author	Normoblasts				Megaloblasts				Normoblast (total %)	Megaloblast (total %)	Myeloid (total %)	R.E.S. Cells (%)	Monocytes	Lymphocytes	Megakaryocytes
	Pronormoblast	Basophilic	Polychromatic	Orthochromatic	Pronormoblast	Basophilic	Polychromatic	Orthochromatic							
Daniachij (1936)		0·6	15·0			0·6			15·6	0·6	72·9	0·6	1·0	9·3	—
Hansen (1938) ..		23·4			—	—	—	—	26·7	—	62·0	0·5	1·8	7·7	0·6
Pitts and Packham (1939)	0·2	2·8	5·3	2·4	—	—	—	—	10·7	—	63·2	—	0·7	12·0	—
Callender (1946)	0·6	2·4	12·8	7·0	—	—	—	—	22·8	—	68·7	2·2	1·0	4·3	—
Gómez (1947) ..									25·05						
Mild anaemias, present series ..	0·5	1·5	7·4	5·2	—	—	—	—	15·4	—	73·0	3·0	1·0	7·4	—

figures show the lowest percentage of normoblasts, but when they made their sternal punctures they withdrew 10 ml. and probably diluted their specimens with blood. They also included some patients with anaemia in their series, since they reported minimum haemoglobin values of 8.97 g. %.

Apart from the finding of megaloblasts our figures for the mild normoblastic anaemias are probably nearer Daniachij's (1936). Compared with Callender's (1946) nine cases examined during the third trimester and those of Montoya Gómez (1947) and Hansen (1938), there appears to be less hyperplasia of the erythroid elements.

The degree of hyperplasia and the stage of red cell development at which it occurs are the most striking features in these cases of anaemia. As a control the

figures may be compared with the values found in four cases of post-haemorrhagic anaemia. These four patients were essentially healthy and their anaemia was entirely due to blood loss. The marrow counts are given at the foot of Table I. The presence of mitotic activity in the marrow and of reticulocytes both in the marrow and in the peripheral blood may be taken as evidence of hyperplasia. In addition the reticulocytosis indicates that there is no interference with maturation, and, as one would expect, the highest percentage of nucleated red cells occurs in the orthochromatic normoblast type. When, however, primary or dyshaemopoietic anaemia occurs the picture is altered. In Group 1 there is a reduction in the percentage of normoblasts, most marked in the orthochromatic type, with a tendency to increase in the earlier forms. As the anaemia increases in severity the changes and tendencies become more marked. A marked increase in the percentage of normoblasts occurs in Group 2, but this increase is entirely confined to the phases before the orthochromatic, especially the basophilic and polychromatic. At this stage in the anaemic process there was also an increase in the number of macronormoblasts, particularly the more mature forms. A new type of cell, the megaloblast, makes its appearance in Group 3, and with it the percentage of normoblasts becomes diminished although less markedly in the earlier forms. Macronormoblasts, on the other hand, have undergone a further increase and for the first time plasma cells and reticulum cells showed a tendency to increase. An even greater reduction in the normoblastic series occurred in Group 4. Macronormoblasts were also diminished although the earlier forms were increased. Megaloblasts were greatly increased and a similar marked increase occurred in the plasma and reticulum cell series.

From the above it will be apparent that as the anaemic process increases in severity the percentage values for each cell series follow a curve. The peak in each curve, however, occurs at a different level of haemoglobin, the normoblast preceding the macronormoblast, which in turn occurs earlier than the megaloblast. This process can be rendered in graphic form as in Fig. 2. It may be said that with increasing anaemia successive waves of hyperplasia occur in the marrow affecting the normoblast first, then the macronormoblast, and finally the megaloblast. More detailed examination of the results indicates that changes of a similar nature occur within each cell series. As the anaemia increases in severity the hyperplasia occurs at a progressively earlier stage. This may be stated in another and possibly more accurate way by saying that increase in severity of the anaemia is associated with a slowing of maturation at an earlier stage of differentiation. Reference to the figures given for the total percentage of nucleated red cells indicates that, while there is some evidence of a true hyperplasia occurring in Group 2 anaemias, this is not apparent in the other groups. In this respect calcu-

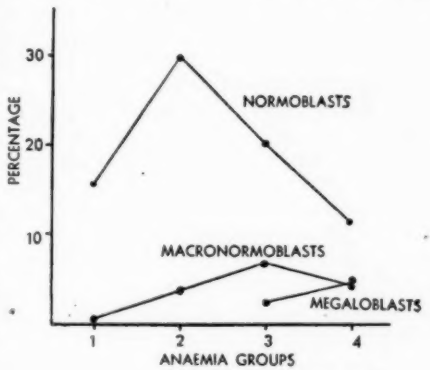


FIG. 2

lation of percentages is misleading. An increase in the number of fat cells is a common finding in marrow smears from cases of very severe anaemia, and would tend to indicate a decrease in the number of haemopoietic foci. Apart from this it seems probable that there is some relationship between these groups of anaemias and that the megaloblastic type is merely one phase in the anaemic process.

Certain points still remain for discussion although few conclusions can be drawn from them. It will be noted that we found reticulocytes frequently in the marrow smears from cases of severe anaemia although none were present in the peripheral blood. Two possible explanations may be given. These reticulocytes may be abnormal cells. Alternatively their release into the peripheral blood may be conditioned by the rate of maturation of the nucleated red cells.

In any hyperplastic process one might expect information from an estimation of mitotic activity, but in marrow smears it is almost impossible to determine which type of red cell is undergoing mitosis. Changes were noted in the incidence of mitosis, but little significance can be attached to them.

So far we have not mentioned the abnormal erythroblast, type b. In appearance abnormal erythroblasts seemed to be an intermediate form between the normoblast and megaloblast. They were found in almost all marrow smears, but were most common in Group 4. It is possible that this cell has been mistaken for a megaloblast and this may account for communications reporting the presence of the latter cell in normal pregnancy marrows.

From this survey it would appear that, coincidental with the appearance of anaemia, there is a slowing of red cell maturation, and, as the severity of the process increases, maturation is slowed at progressively earlier phases. Apparent proliferation occurs in the stages before that at which maturation is halted. Cases showing hyperplasia even with a " shift to the left " as in Group 2 generally respond to iron. Where, however, the " shift to the left " is accompanied by relative inactivity in the erythroblast series the condition is likely to prove resistant to iron therapy. The apparent relationship between these various types of anaemia would suggest an upset in metabolism rather than a primary defect in the haemopoietic system.

Summary

The bone marrow has been studied in 46 cases of anaemia of pregnancy. In 28 the anaemia was of iron-deficiency type. The remaining cases were of pernicious variety.

In iron-deficiency anaemias marrows are entirely normoblastic. Main features are slowing of maturation, directly related to the severity of the anaemia, and hyperplasia at progressively earlier phases, that is a " shift to the left." As the anaemia increases in severity abnormal early erythroblasts, macronormoblasts, and reticulum cells become more common.

In pernicious anaemia of pregnancy the marrow is dimorphic and the peripheral red cells may be either microcytic or macrocytic.

Where the peripheral blood is microcytic there are few megaloblasts in the marrow. Normoblasts show a greater " shift to the left " and there is less evidence of hyperplasia in this cell-series. Macronormoblasts are much increased and both reticulum and plasma cells are common.

The microcytic megaloblastic anaemias show a partial response to iron, but normal blood values are only obtained if folic acid or liver is given.

In the macrocytic variety megaloblasts are common in the marrow. There is a diminution in the total number of normoblasts, although the "shift to the left" in this series is even more marked. A "shift to the left" is also apparent in the macronormoblasts. Reticulum and plasma cells are greatly increased. Patients with this variety show no response to iron.

It is suggested that these various types of anaemia are merely phases of the same pathological process and that there is an aetiological relationship between them.

The results would indicate that hyperplasia occurs in the red cell series as a result of slowing of maturation and is first apparent in the normoblasts, subsequently affects macronormoblasts, and finally megaloblasts as the anaemic process increases in severity.

Abnormal early erythroblasts may cause difficulty in interpretation and these cells may be mistaken for megaloblasts.

A statistical analysis of our results has been made and a copy of our protocols giving the details has been lodged with the Librarian of the British Museum. We would like to thank Dr. Robb, of the Mathematics Department, Glasgow University, for his help and guidance in the analysis.

We should like to thank British Schering, Ltd., and Bengers, Ltd., for supplies of intravenous iron used in the treatment of anaemias.

References

Abramson, L. (1938). *Acta med. scand.*, **96**, 319.
Beckman, M. (1921). *Mschr. Geburtsh. Gynäk.*, **56**, 119. Quoted by Callender (1944).
Callender, S. T. E. (1944). *Quart. J. Med.*, **13**, 75.
—— (1946). *J. Path. Bact.*, **58**, 580.
Daniachij, M. A. (1936). *Zbl. Gynäk.*, **60**, 1220.
Davidson, L. S. P., Davis, L. J., and Innes, J. (1942a). *Brit. med. J.*, **2**, 31.
—— —— —— (1942b). *Quart. J. Med.*, **11**, 19.
Forssell, J. (1939). *Acta med. scand.*, Suppl. 101.
Hansen, R. (1938). *Z. Geburtsh. Gynäk.*, **116**, 398.
Leitner, St. J. (1948). *Acta med. scand.*, **130**, 66.
Mallarmé, J. (1948). *Blood*, **3**, 103.
Markoff, N. (1939). *Z. Geburtsh. Gynäk.*, **119**, 13.
Montoya Gómez, M. (1947). *Clin. y Lab.*, **44**, 170.
Pitts, H. H., and Packham, E. A. (1939). *Arch. intern. Med.*, **64**, 471.
Rohr, K. (1940). *Das menschliche Knochenmark*. Leipzig.
Russo, F. (1937). *Ann. Ostet. Ginec.*, **59**, 1169.
Scott, J. M., and Govan, A. D. T. (1949). *Brit. med. J.*, **2**, 1083.
Segerdahl, E. (1941). *Acta med. scand.*, **108**, 483.
Stevenson, E. M. K. (1938). *Trans. Edinb. obstet. Soc.*, **58**, 81.
Whitby, L. E. H., and Britton, C. J. C. (1946). *Disorders of the Blood*, 5th ed. London.
Wolff, J. R., and Limarzi, L. R. (1946). *Amer. J. Obstet. Gynec.*, **51**, 447.

J. clin. Path. (1952), **5**, 154.

THE DIAGNOSIS OF CARRIERS OF COOLEY'S ANAEMIA

BY

FRANCIS S. MOONEY

From the British Military Hospital, Malta

(RECEIVED FOR PUBLICATION JUNE 21, 1951)

Some 13 years ago Angelini (1937) and Caminopetros (1938a and b) independently postulated the existence of a mild or carrier form of Cooley's anaemia, basing their deductions on the discovery of the frequent occurrence of an increased erythrocytic resistance to saline in certain healthy relatives of clinical cases of this anaemia.

Since then the hereditary and familial nature of the disease has become widely accepted ; nevertheless observers still differ in their opinions regarding the best way of detecting the latent or carrier form. Smith (1942) believes that " the occurrence of morphological changes (in the erythrocyte) constitutes a fundamental principle in the detection of the mild trait," and that, although erythrocytic saline resistance was the most reliable single investigation, a normal result would not necessarily invalidate the diagnosis.

Valentine and Neel (1944) agree that the saline resistance may not be so dramatically altered, and believe that the diagnosis rests upon several items, including a low mean cell volume, the presence of stipple cells, resistance to iron therapy, and a relatively normal mean cell haemoglobin concentration.

Dameshek (1946) attaches great importance for the detection of the mild trait to the presence of target or oval cells, and the relationship of haemoglobin content, erythrocyte count, and haematocrit. This relationship is not specifically defined, and he concedes that in many cases " it may not be dramatically abnormal." It would appear, then, that although many carriers present a clearly defined haematological picture, particularly when the erythrocytic resistance to saline is high, there still remains a number of individuals in whom the diagnosis tends to become a matter of personal interpretation of inconstant, and often complex, relationships.

In this paper 13 healthy Maltese relatives of a patient with severe Cooley's anaemia previously described (Mooney, 1951), have been examined, and the haematological findings analysed in an attempt to define the carrier state more accurately and to discover a reliable method of detecting its presence.

Family History

The paternal and maternal genealogical trees are shown in Fig. 1. The paternal great-grandparents were both of pure Maltese ancestry, and seven children were born of the marriage, four boys and three girls. One of the boys died at the age of 15 after many years of indefinite ill-health. The cause of his death is obscure. The remaining six children were healthy in all respects, and the eldest boy became the grandparent of the family under scrutiny. It will be seen that he married twice. His first wife died in 1919 from influenza, his second in 1946 from a cancer of the colon. Nine children

were born of the first marriage, but only two have survived beyond infancy. These are Mr. B. (father) and J. B. (uncle). The second marriage was to a first cousin, and, although there were 13 children, only three survived beyond the second year of life.

The symptoms manifested by the 17 children who died in infancy were, according to their father and surviving brothers, strikingly similar. All were pale and listless, failed to gain weight, and tended to develop a progressive general weakness which relentlessly deteriorated, until finally death supervened. None survived beyond the age of 2. Whether the deaths were, in fact, all due to Cooley's anaemia is a matter for conjecture. As the second marriage was to a close relative, it seems reasonable to assume that, in the children of this marriage at any rate, Cooley's anaemia may have played a large part in their deaths. In the first marriage, however, it is more problematical. Certainly it still remains a possibility, as fortuitous consanguinity in marriage is inevitably high in a small island community such as Malta.

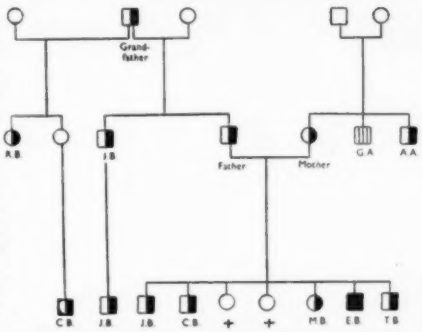


FIG. 1.—Genealogical tree of the affected family.

Mr. B., the father of the family, married 14 years ago and had seven children. Of these, two died from a severe type of anaemia before this investigation, another is a clinical case of Cooley's anaemia (Mooney, 1951), and the remainder are quite healthy.

J. B. (uncle) is married and has had two children. The first child died from dysentery when 2 weeks old. The second is J. B. (cousin). Of the three surviving children of the grandfather's second marriage, only J. B. (aunt) is married. She has had four children, all of whom are healthy, and the youngest, C. B. (cousin), is one of the individuals who has been scrutinized.

Maternal Origin of Family.—This half of the family is also of pure Maltese descent. The grandfather died at the age of 43 from diabetes, and the grandmother at the age of 66 after an accident. There were eight children of the marriage, four boys and four girls. One girl died from enteric fever in infancy, the second became the mother of the family under scrutiny, the third is married and has had 13 children, all of whom are healthy, and the fourth is unmarried.

The four brothers are all alive. The eldest, A. A. (uncle), is married and has had four children. Two of these were twins who died in infancy from dysentery, and the other two were both girls, who are now also married. The second brother, G. A., is married, but has no children. The remaining two sons were both serving in the Navy and were abroad during this investigation.

Investigations

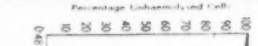
Routine examination of blood included a full count, a differential white cell count, calculating various erythrocytic indices, estimating serum bilirubin, and the titre of Brucella antibodies, and the Kahn reaction for syphilis. In all but four members of the family a special study was made of the reticulocytosis and stippling of the red cells.

Red Cell Count.—An accurate red cell count is notoriously difficult to achieve, and various investigators have estimated the coefficient of variation to be as high as 7–8% (Magath, Berkson, and Hurn, 1936; Berg, 1945). In order to minimize this error and consequently obtain more accurate estimations of the mean cell thickness, diameter,

TABLE I

RESULTS OF HAEMATOLOGICAL INVESTIGATIONS

Name	Age	R.B.C.s per c.mm.	Hb. % (100% = 14·5 g.)	Colour Index	Mean Corpuscular Haemoglobin Concentration (%)	Mean Cell Volume (cμ)	Mean Cell Diameter (μ)	Mean Cell Thickness (μ)	Stipple Cells (per c.mm.)	Reticulocytes (per c.mm.)	Target Cells	Standard Deviation in Size of R.B.C.s (σ)	Titre of *Brucella melitensis* Antibody
Grandfather	66	4,970,000	87	0·88	32·3	78·5	7·71	1·69	19,500	—	Present	0·675	Nil
Father ..	33	5,520,000	87	0·79	28·7	79·6	7·52	1·79	6,000	32,000	,,	0·502	,,
J.B. (brother)	12	5,040,000	80	0·79	29·7	77·3	7·39	1·80	18,000	60,000	Absent	0·545	,,
C.B. (brother)	10	4,930,000	87	0·88	30·8	81·1	7·17	2·00	9,500	50,000	Present	0·570	1/50
M.B. (sister)	4	4,660,000	87	0·94	29·3	90·0	7·54	2·03	3,000	24,000	Absent	0·462	Nil
T.B. (brother)	1	4,970,000	84	0·86	—	—	—	—	5,000	24,000	,,	—	—
J.B. (uncle)	38	5,170,000	91	0·88	33·0	77·3	7·24	1·92	14,000	—	Present	0·560	Nil
J.B. (cousin)	12	4,560,000	87	0·95	33·6	82·2	7·49	1·90	5,000	33,000	Absent	0·587	,,
R.B. (aunt)	16	5,000,000	82	0·82	33·0	73·0	7·56	1·63	2,000	—	Present	0·744	1/25
C.B. (cousin)	4	4,870,000	80	0·82	31·2	70·8	7·28	1·70	7,000	—	,,	0·735	Nil
A.A. (uncle)	52	5,000,000	96	0·96	32·8	85·0	7·60	1·87	6,000	32,000	Absent	0·547	1/25
G.A. (uncle)	36	5,300,000	104	0·98	31·5	87·0	7·40	2·08	<250	25,000	,,	0·440	Nil
Mother ..	36	4,390,000	81	0·94	30·9	86·6	7·28	2·03	3,000	36,000	,,	0·478	1/25



and volume, two separate counts were done by a technician and myself, and instead of the usual 80 small squares 160 were counted in each case. The mean of the counts was taken as correct, and by this means the coefficient of variation was reduced to approximately ±3%.

Stipple Cell Count.—The method employed was that recommended by Whitby and Britton (1946), using a simple methylene blue stain, and is considered to be superior to the ordinary Leishman film in the search for stipple cells.

The remaining investigations were performed by the commonly accepted techniques. Fully oxygenated blood was used in the estimation of the mean cell volume.

Differential Saline Resistance Test (Quantitative Fragility Test).—As Creed (1938) has pointed out, fragility varies considerably with the degree of oxygen saturation, so fully oxygenated blood was used in this estimation. The technique was based upon that of Creed (1938), except that a range of concentrations of 0.48% to 0.16% saline was employed in preference to the more usual 0.72% to 0.28%. It was also found that a more easily readable graph was obtained by plotting the percentage of unhaemolysed cells as abscissae against saline concentration.

Price-Jones Curve.—As no elaborate apparatus was available for this estimation, the simplified apparatus described by Humble and Belyavin (1948) was used. The films were stained by 1% eosin for one minute after preliminary fixation in methyl alcohol.

The remainder of the investigations were performed by the usual techniques. Fully oxygenated blood was used in the estimation of the mean cell volume.

Results

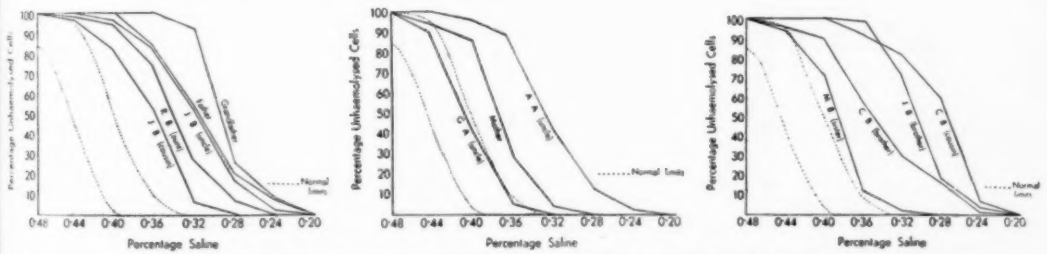
The results are most conveniently expressed in the tables and graphs. Table I is a simple record of the various haematological indices obtained. Table II reveals the number of abnormal readings found in each individual.

The results of the differential or quantitative fragility tests are depicted in Figs. 2, 3, and 4. The Kahn test results and serum bilirubin were normal in each case.

Discussion

From Table II it is clear that G. A. is the only member of the family examined in whom all investigations are normal. The remaining individuals reveal at least three abnormal indices, and an increase in both erythrocytic resistance to saline and the stipple cell count is common to all.

Fragility of Erythrocytes.—Many observers have recorded examples of normal erythrocyte fragility in both mild and severe forms of the disease (Cooley, 1928 ;



FIGS. 2, 3, and 4.—Results of quantitative saline resistance tests in members of the family.

TABLE II

NUMBER OF ABNORMAL READINGS IN CERTAIN MEMBERS OF THE FAMILY

	Mean Cell Diameter	Mean Cell Volume	Mean Cell Thickness	Target Cells	Stipple Cell Count	Quantitative Erythrocyte Saline Resistance	Standard Deviation in Size of R.B.C.s
Grandfather	+	N	—	+	+	+	+
Father	+	N	—	+	+	+	+
J.B. (brother) ..	+	—	—	N	+	+	+
C.B. (brother) ..	N	N	N	+	+	+	+
M.B. (sister)	+	N	N	N	+	+	N
J.B. (uncle)	N	—	N	+	+	+	+
J.B. (cousin)	+	N	N	N	+	+	+
R.B. (aunt)	+	—	—	+	+	+	+
C.B. (cousin)	N	—	—	+	+	+	+
Mother	N	N	N	N	+	+	N
A.A. (uncle)	+	N	N	N	+	+	+
G.A. (uncle)	N	N	N	N	N	N	N

N = result was within normal limits; + = result above normal limits; — = result below normal limits.

Baty, Blackfan, and Diamond, 1932; Smith, 1942; Valentine and Neel, 1944; Dameshek, 1946). In each of these, however, the result was invariably expressed merely as a record of the beginning and end-points of haemolysis. In this series the fragility tests of both Mrs. B. and M. B. (sister), if expressed in these terms, would certainly have been reported within normal limits. Nevertheless, by a more critical quantitative method, a significant increase in the resistance becomes apparent in both (Figs. 3 and 4).

Stipple Cells.—The occurrence of stipple cells in severe anaemia and leukaemia is well recognized, but their presence in healthy individuals is almost entirely confined to asymptomatic cases of mild plumbism. The constant appearance of stipple cells here is, therefore, of considerable significance. Furthermore, as this cell has also been reported in severe cases of Cooley's anaemia by many observers (Cooley, 1928 and 1929; Haden, 1934; Dameshek, 1940; Neel and Valentine, 1945), there would appear to be grounds for the belief that its presence indicates the existence of some factor common to both major and minor Cooley syndromes. The nature of the stipple cell itself has given rise to some speculation. Although most observers agree that it is immature, and closely related to the reticulocyte, Smith (1942) believed it to be a degenerate erythrocyte. As the stipple cell occurs in symptomless carriers with a relatively normal count, he believed it unlikely that there would be evidence of regeneration in the peripheral blood of such cases. Nevertheless, the weight of evidence is against this view. Whitby and Britton (1933) established a close relationship between the sum of polychromatic and stipple cells and the reticulocytes. Rosegger (1936), too, has shown that stippling, polychromasia, and reticulum have similar iso-electric points. The data from this family support a relationship between the stipple cell and reticulocyte in this condition.

Mean Cell Volume, Diameter, and Standard Deviation in Size.—It will be seen that the average M.C.V. is inclined to be on the low side of normal, while the

average M.C.D. tends to be high. These figures, although of no absolute diagnostic value, give a certain amount of information about the usual morphology of the erythrocyte in the carriers of this anaemia. Similarly, although the standard deviation in the size of the erythrocyte is of little specificity from a diagnostic point of view, nevertheless it will be seen that in the majority of cases (75%) it is raised.

Target Cells.—Target cells have long been thought to be of profound significance in Cooley's anaemia, largely due to the work of Dameshek (1943), who regarded them as congenitally defective cells, and believed their presence to be essential in both mild and severe forms of this anaemia. Target cells, however, were first described in a case of sickle-cell anaemia (Haden and Evans, 1937), and Barrett (1938) in his monograph records them in a variety of conditions, including obstructive jaundice with or without anaemia, in idiopathic steatorrhoea, after splenectomy (whatever the cause of the operation), and in a variety of anaemias whenever the colour index falls below 0.8. They have been recognized more recently (Valentine and Neel, 1944) in cases of Laennec's cirrhosis and cardiac decompensation, and I have found them occasionally in cases of untreated pernicious anaemia. In the present investigation, target cells were present in less than half of the cases, and although they are undoubtedly common in Cooley's anaemia, particularly in the severe form, they can hardly be regarded as either pathognomonic or as a *sine qua non* in mildly affected individuals.

Mean Cell Thickness.—Although every member of the family, except G. A., shows a low colour index, the mean corpuscular haemoglobin concentration is normal. This relationship, combined with the large cell diameter and low volume mentioned above, would appear to indicate a low mean cell thickness. It is surprising, therefore, to discover that this index falls below the normally accepted limits of 1.8 μ to 2.3 μ in only five individuals (Table I). The explanation may be in the limits themselves: thus a "normal" cell with a thickness of 1.8 μ must also possess a volume of 78 cμ and a diameter of 7.4 μ, a most unusual combination in a healthy individual. What appears to be of greater significance is the discovery that the mean erythrocyte thickness in all, except G. A., lies below the average of 2.15 μ. This coincides with the views of Bradford and Dye (1936), who, from a study of five severe cases of Cooley's anaemia, concluded that leptocytosis was probably the fundamental defect in that condition.

From these findings it is clear that the most consistently recurring abnormalities in the blood picture of the individuals examined are the increased stipple cell count and quantitative saline resistance. Indeed, in only one individual (G. A.) are these normal, and in this single case all other indices are also normal. Both these indices have been found to be raised in all severe cases, whenever they have been estimated. It would appear, therefore, that as these findings are rare in other types of anaemia, their presence in many of the relatives of sufferers from Cooley's anaemia is in itself strong *a priori* evidence for assuming the existence of the carrier state in those persons.

None of the other indices reveals such constant abnormalities, and yet, on the assumption that all but one of the individuals examined are, in fact, carriers, it is possible to deduce a considerable amount of information about the usual morphology of the erythrocyte in this condition. Thus, the cell thickness would appear

to be always below average, the diameter to be usually increased, and the volume low. Target cells may or may not be present, and there is usually a mild accompanying degree of anisocytosis. In all cases there is a subclinical anaemia, in which the colour index is below unity, but the mean corpuscular haemoglobin concentration remains relatively normal. These indices, however, are subject to wide fluctuations in any one case, and at the best represent the average picture.

Summary

The haematological findings in 13 healthy relatives of a case of Cooley's anaemia have been analysed in an attempt to discover a simple means of detecting the presence of the mild trait.

In only one individual were normal readings found in all haematological investigations.

An increased stipple cell count and raised quantitative erythrocytic resistance to saline were found in all the remaining individuals, and it is submitted that by means of these two tests carriers of the trait may be detected.

The haematological picture usually found in the mild trait is outlined.

It is a pleasure to acknowledge my gratitude to the Deputy Director of Pathology, Middle East Land Forces, for permission to present this work, and to Ptes. Long and Anderson for their technical assistance.

REFERENCES

Angelini, V. (1937). *Minerva med., Torino*, **2**, 331.
Barrett, A. M. (1938). *J. Path. Bact.*, **46**, 603.
Baty, J. M., Blackfan, K. D., and Diamond, L. K. (1932). *Amer. J. Dis. Child.*, **43**, 667.
Berg, W. N. (1945). *Amer. Rev. Tuberc.*, **52**, 179.
Bradford, W. L., and Dye, J. (1936). *J. Pediat.*, **9**, 312.
Caminopetros, J. (1938a). *Ann. Méd.*, **43**, 27.
—— (1938b). Ibid., **43**, 104.
Cooley, T. B. (1928). *Amer. J. Dis. Child.*, **36**, 1257.
—— (1929). *Amer. J. Dis. Child.*, **38**, 103.
Creed, E. ff. (1938). *J: Path. Bact.*, **46**, 331.
Dameshek, W. (1940). *Amer. J. med. Sci.*, **200**, 445.
—— (1943). Ibid., **205**, 643.
—— (1946). *New. Engl. J. med.*, **234**, 829.
Haden, R. L. (1934). *Amer. J. med. Sci.*, **188**, 441.
—— and Evans, F. D. (1937). *Arch. intern. Med.*, **60**, 133.
Humble, J. G., and Belyavin, G. (1948). *Journal of Clinical Pathology*, **1**, 77.
Mooney, F. S. (1951). *Arch. Dis. Childh.*, **26**, 231.
Magath, T. B., Berkson, J., and Hurn, M. (1936). *Amer. J. clin. Path.*, **6**, 568.
Neel, J. V., and Valentine, W. N. (1945). *Amer. J. med. Sci.*, **209**, 568.
Rosegger, H. (1936). *Klin. Wschr.*, **15**, 158.
Smith, C. H. (1942). *J. Pediat.*, **20**, 370.
Valentine, W. N., and Neel, J. V. (1944). *Arch. intern. Med.*, **74**, 185.
Whitby, L. E. H., and Britton, C. J. C. (1933). *Lancet*, **1**, 1173.
—— —— (1946). *Disorders of the Blood*, 5th ed. London.

J. clin. Path. (1952), **5**, 161.

STARCH-FERMENTING, GELATIN-LIQUEFYING CORYNEBACTERIA AND THEIR DIFFERENTIATION FROM *C. DIPHTHERIAE GRAVIS*

BY

G. T. COOK AND W. H. H. JEBB

From the Public Health Laboratory, Oxford

(RECEIVED FOR PUBLICATION DECEMBER 4, 1951)

During recent years attention has been drawn to the isolation of starch-fermenting, gelatin-liquefying corynebacteria from acute cases of sore throat (Jebb, 1948; Saxholm, 1951). Fourteen strains have been isolated in this laboratory between January, 1946, and June, 1951, 12 from acute tonsillar infection, one from the throat of a symptomless carrier, and one from a lesion on the hand. In addition, a further 31 strains from other laboratories have been investigated by one of us (W. H. H. J.). With the decrease in the number of cases of *gravis* diphtheria these 14 strains formed a considerable proportion (35%) of all starch-fermenting corynebacteria isolated in this laboratory during the last five years (Table I). The strains can be distinguished from *C. diphtheriae* both biochemically and because they produce two toxins, only

TABLE I

NUMBER OF STRAINS OF *C. diphtheriae gravis* AND STARCH-FERMENTING, GELATIN-LIQUEFYING CORYNEBACTERIA ISOLATED BETWEEN JANUARY, 1946, AND JUNE, 1951

Year	Strains of *C. diphtheriae gravis*	Starch-fermenting, Gelatin-liquefying Strains
1946	15*	3
1947	5	4
1948	3	2
1949	2	1
1950	1	2
1951 (Jan.–June)	0	2
Total	26*	14

* Including seven strains isolated from two small outbreaks.

one of which is neutralizable by diphtheria antitoxin (Petrie and McClean, 1934), but as they may be confused with *C. diphtheriae gravis* their correct and rapid identification is a matter of some interest and importance.

Until recently this identification has taken some time; thus gelatin liquefaction may take up to 10 days, trehalose fermentation up to 16 days (Jebb, 1948), and nitrate broth is commonly incubated for five days before testing by the Griess-Ilosvay method (Wilson and Miles, 1946). The most rapid method in use heretofore

has been intradermal inoculation into protected and unprotected guinea-pigs (Jebb, 1948), but even this, allowing time for the preparation of test and control cultures and the development of the lesion, may take several days.

Diagnostic Methods

All strains of starch-fermenting, gelatin-liquefying corynebacteria tested by us for urea hydrolysis and nitrate reduction have split urea rapidly and have failed to reduce nitrates; similar results have been reported by Saxholm (1951). *Gravis* strains, on the other hand, are generally considered to reduce nitrates but not to hydrolyse urea; all such strains tested by us have behaved in this way. Tests for urea hydrolysis and nitrate reduction provide a rapid method for distinguishing these two organisms. By the use of a urea slope (Christensen, 1946) and a nitrate strip on a blood agar plate (Cook, 1950) an indication of the nature of the culture being tested can be obtained within 18 hours and frequently within six hours.

In view of the atypical colonial appearances sometimes shown by corynebacteria it is suggested that the following procedure be followed with all organisms which on primary isolation colonially and morphologically appear to be diphtheria bacilli. A single colony is picked from the tellurite agar or blood agar plate to a Löffler serum slope. The slope is incubated for six to eight hours and then are inoculated (1) a blood agar plate (for purity, haemolysis, and colonial appearances), (2) starch, glucose, maltose and sucrose serum-water "sugars," and serum broth, (3) a urea slope, and (4) a second blood agar plate to which a nitrate strip is then added. The reactions of the three types of *C. aiphtheriae* and of the starch-fermenting, gelatin-liquefying strains are summarized in Table II. The results of urea hydrolysis and nitrate reduction will be available after overnight incubation and will form a basis for further investigations, though some of the fermentation reactions may

TABLE II

RAPID BIOCHEMICAL REACTIONS OF THE THREE TYPES OF *C. diphtheriae* AND OF STARCH-FERMENTING, GELATIN-LIQUEFYING STRAINS

Strain	Fermentation Reactions				Nitrate Reduction	Urea Hydrolysis
	Glucose	Maltose	Sucrose	Starch		
C. diphtheriae mitis	AC	AC	—	—	usually+	—
C. diphtheriae intermedius	AC	AC	—	—	+	—
C. diphtheriae gravis	AC	AC	—	AC	+	—
Starch-fermenting, gelatin-liquefying strains	AC	AC	—	AC	—	+

AC = acid and clot; + = positive reaction; — = no fermentation or negative reaction.

occasionally take rather longer to develop. If the organism appears to be a *mitis* strain of *C. diphtheriae* subcutaneous virulence tests can then be performed in the usual way, while with *intermedius* and *gravis* strains isolated from clinical cases no further action will probably be necessary. Starch-fermenting strains which do not hydrolyse urea and yet colonially and morphologically do not appear to be typical *gravis* strains should be tested for virulence by subcutaneous guinea-pig inoculation. Starch-fermenting strains which hydrolyse urea should always be further investigated

by intradermal inoculation into protected and unprotected guinea-pigs and possibly by testing for gelatin liquefaction and trehalose fermentation.

Many starch-fermenting, gelatin-liquefying strains produce two toxins, one of which is neutralizable by diphtheria antitoxin and the other resembles that of *C. ovis*. It is, therefore, important that animal inoculations with these strains be performed by the intradermal route, as the results of the classical subcutaneous virulence test may be misleading. If the subcutaneous route is used the unprotected guinea-pig may die within 24 to 48 hours with the post-mortem appearances of diphtheria infection, while 500 units of diphtheria antitoxin may protect the other guinea-pig indefinitely (Mair, 1928). If death should supervene, the post-mortem appearances are not characteristic of diphtheria but resemble those of infection with *C. ovis* (Barratt, 1933 ; Petrie and McClean, 1934). On intradermal injection, however, starch-fermenting, gelatin-liquefying strains produce a characteristic ulcerating lesion which is not neutralized by 1,000 units of diphtheria antitoxin.

Discussion

The pathogenicity and epidemiological significance of starch-fermenting, gelatin-liquefying strains are still in doubt, but their more frequent recognition in recent years is of interest. It is possible that many of them are regarded as atypical *gravis* strains and that their incidence is greater than is generally realized. Morphologically and colonially they may resemble *C. diphtheriae gravis*, and the fermentation reactions of both organisms in the usual diagnostic " sugars " are identical. Virulence tests are frequently omitted with *gravis* strains, but when they are done the subcutaneous method is usually chosen ; as has already been stated starch-fermenting, gelatin-liquefying organisms may also be expected to kill an unprotected guinea-pig on subcutaneous inoculation with the production of post-mortem changes characteristic of diphtheria. It is suggested that the routine use of tests for urea hydrolysis and nitrate reduction with all starch-fermenting corynebacteria may help to identify these strains and so reveal their true incidence.

Most of the starch-fermenting, gelatin-liquefying cultures examined by us have been isolated during the summer months from sporadic cases or carriers among children or adults living in rural areas. This pattern of distribution may perhaps be related to the consumption of raw milk, and we have had evidence of the association of these organisms with milk on three separate occasions. In one infected family the organism was cultivated from the milk bottle which had contained raw milk supplied to the household. On another occasion the organism was isolated from the milk of a cow suffering from mastitis. Recently we have also isolated a strain from an indolent ulcer on the hand of a milker.

In general, infection with these organisms clinically resembles tonsillitis rather than diphtheria, though some membrane formation in the throat may be seen. The disease is usually mild, but the severity varies from one patient to another ; this may be an expression of the amount of diphtheria toxin produced by the particular strain and the state of immunity of the individual. Epidemics of this disease are unusual and most of the 45 strains investigated by us were isolated from single cases. As many of these strains elaborate a toxin which is neutralizable in part at least by diphtheria antitoxin (Petrie and McClean, 1934), it would appear to be wise to regard patients infected with these organisms as suffering from true diphtheria.

Summary

Starch-fermenting, gelatin-liquefying corynebacteria have formed 35.0% of all starch-fermenting corynebacteria isolated at this laboratory during the past five years.

These strains can be rapidly distinguished from *C. diphtheriae gravis* by their ability to hydrolyse urea and their failure to reduce nitrates. The inclusion of tests for urea hydrolysis and nitrate reduction in the routine examination of all suspected diphtheria organisms is suggested. Starch-fermenting corynebacteria which hydrolyse urea and fail to reduce nitrates should be examined for trehalose fermentation and gelatin liquefaction and inoculated intradermally into protected and unprotected guinea-pigs. It is important to use the intradermal route, as the results of subcutaneous inoculation may be misleading.

REFERENCES

Barratt, M. M. (1933). *J. Path. Bact.*, **36**, 369.
Christensen, W. B. (1946). *J. Bact.*, **52**, 461.
Cook, G. T. (1950). *Journal of Clinical Pathology*, **3**, 359.
Jebb, W. H. H. (1948). *J. Path. Bact.*, **60**, 403.
Mair, W. (1928). Ibid., **31**, 136.
Petrie, G. F., and McClean, D. (1934). Ibid., **39**, 635.
Saxholm, R. (1951). Ibid., **63**, 303.
Wilson, G. S., and Miles, A. A. (1946). *Topley and Wilson's Principles of Bacteriology and Immunity*, 3rd ed., p. 368. London.

J. clin. Path. (1952), **5**, 165.

THE ANTIBIOTIC SENSITIVITY OF PATHOGENIC STAPHYLOCOCCI

BY

W. D. LINSELL

From the Department of Bacteriology, St. Mary's Hospital Medical School, London

(RECEIVED FOR PUBLICATION NOVEMBER 23, 1951)

Recent evidence indicates that the pathogenic bacteria are continuously changing in their susceptibility to antibiotics that have come into common clinical use. The occurrence of penicillin-resistant staphylococci in human infection has become commonplace (Anderson, Howard, and Rammelkamp, 1944; Spink, Ferris, and Vivino, 1944; Blair, Carr, and Buchman, 1946). This is thought by many to be due to the selective survival of penicillinase-producing variants during treatment (Demerec, 1945), although infection with two types of staphylococci or the occurrence of secondary infection with penicillin-resistant strains may also play a part.

The result has been a steadily increasing incidence of penicillin-resistant staphylococci in the hospital environment (Barber and Rozwadowska-Dowzenko, 1948; Forbes, 1949). Evidence has also been produced of an increased incidence of streptomycin-resistant staphylococci in hospital practice (Rountree, Barbour, and Thomson, 1951).

New antibiotics highly effective against staphylococci may bring the same change in average sensitivity, and it is the purpose of this paper to indicate the present position with regard to the newer antibiotics, in particular terramycin and aureomycin. The survey is confined to staphylococci from acute lesions in out-patients. At the same time resistance to penicillin and its relation to previous penicillin treatment was investigated. A carrier of penicillin-sensitive staphylococci might, as a result of penicillin treatment, become a carrier of a resistant strain, and if subsequent autogenous infection occurred the lesion might also yield penicillin-resistant staphylococci.

Material

Most of the strains of coagulase positive staphylococci came from finger and hand infections (60% of isolates), boils, carbuncles, and abscesses of various kinds (33%), the remainder from various skin and ear infections.

Each patient was carefully questioned in regard to previous penicillin treatment. Specimens of pus and swabs, taken from the acute pyogenic lesions of patients attending the hospital casualty department, were inoculated on blood agar, and the coagulase positive cultures were maintained on Dorset egg.

Method

One hundred and eighteen strains were isolated and tested for sensitivity to five antibiotics, using the standard serial dilution method in broth in 3 in. $\times$ $\frac{1}{2}$ in. tubes at *p*H 7.4.

A standard inoculum was used of 10 c.mm. of an overnight broth culture to 10 ml. of broth (approximately 10^6 organisms per ml.). In the case of aureomycin the same technique was employed, but the culture medium was adjusted to *p*H 6.8, as described by Anderson (1951), as this gave more consistent results than at *p*H 7.4, though occasionally there was little difference between the results.

The lowest concentration of antibiotic giving complete inhibition was regarded as the sensitivity level.

Results

In determining the sensitivity of 118 strains of staphylococci to penicillin, strains inhibited by concentrations of 0.25 units were arbitrarily taken as "sensitive" and those growing in concentrations of 0.5 units and above as "resistant." A resistance rate of 18.6% was obtained. Seventy-five per cent of strains were sensitive to 0.062 unit or less.

The findings in regard to resistance to penicillin and a history of previous contact with the antibiotic were analysed. Twelve out of 22 resistant strains (15.8%) came from a group of 76 cases with no previous history of contact with penicillin, whereas nine strains (25%) were obtained from the group of 36 cases previously treated with penicillin. Statistical analysis of this sample shows that $\chi^2 = 1.36$, which for one degree of freedom gives a probability (P) of approximately 0.25. Thus, in this sample, no statistical difference exists between the two groups; in other words, previous treatment with penicillin played no part in deciding whether a new lesion was likely to be caused by a penicillin-resistant staphylococcus.

TABLE I

SENSITIVITY OF 118 STRAINS OF COAGULASE-POSITIVE STAPHYLOCOCCI TO ANTIBIOTICS OTHER THAN PENICILLIN

Concentration (μg. per ml.)	Number of Strains			
	Terramycin	Aureomycin	Streptomycin	Chloramphenicol
32	2	1	2	—
16	—	—	4	1
8	—	2	4	10
4	—	—	90	101
2	—	1	17	5
1	1	72	—	1
0·5	1	42	1	—
0·25	112	—	—	—
0·125	2	—	—	—

Table I summarizes the sensitivity to the principal alternative antibiotics to penicillin. The behaviour of aureomycin and terramycin is very similar except that terramycin is inhibitory at approximately half the concentration of aureomycin weight for weight. Of two strains requiring 32 μg./ml. of terramycin for inhibition, one required 32 μg. and the other 8 μg. of aureomycin for inhibition; both were penicillin-resistant. A third strain sensitive to 1 μg. of terramycin required 8 μg. of aureomycin. All three strains were inhibited by 4 μg. of both chloramphenicol and streptomycin. Table II summarizes the position of individual strains classed

TABLE II

INDIVIDUAL STRAINS RESISTANT TO ANY ONE OF THE NEWER ANTIBIOTICS

Strain Number	Terramycin	Aureomycin	Streptomycin	Chloramphenicol
11*	0·25	0·5	16	4
28*	32	32	4	4
29	32	8	4	4
34B	0·25	0·5	16	4
40*	0·5	1·0	32	4
41	0·25	0·5	32	4
49	0·25	1·0	16	4
81	1·0	8·0	2	4
110	0·25	1·0	16	4
115†	0·25	1·0	4	16

* Penicillin-resistant (10 units). † Penicillin-resistant (0.5 unit).

as resistant to one or more antibiotics. Strain 28 is of particular interest in that it was resistant to penicillin (10 units), terramycin (32 μg.), and aureomycin (32 μg.), being most sensitive to chloramphenicol (4 μg.) and streptomycin (4 μg.). There was no previous record of treatment with aureomycin or terramycin in patients yielding the strains found resistant to these antibiotics.

The two strains found resistant to terramycin were tested for an inhibitor effect. To overnight broth cultures of these and of a sensitive strain, 128 μg./ml. of terramycin was added. All three cultures were incubated for a further 24 hours and Seitz-filtered. The three filtrates were then titrated for terramycin level. No fall in level occurred.

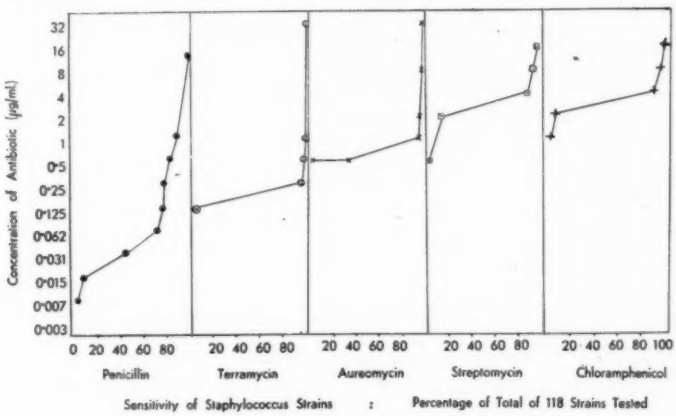


FIG. 1

The complete findings in this series are represented in Fig. 1. It must be emphasized that the penicillin section is displayed in units, not microgrammes, and therefore its superiority weight for weight is much greater than would appear from the figure (1 unit penicillin = 0.0006 mg. crystalline penicillin G).

Discussion

In the present series of strains of staphylococci none of those found resistant to penicillin required more than 10 units for inhibition, and detectable amounts of penicillinase were produced by strains whose resistance was greater than 0.5 unit. This is of interest in view of the highly resistant strains encountered by Spink (1951) in in-patients in whom he found cross-infection occurring with strains of staphylococci of a resistance varying between 500 and 4,000 units.

The sensitivity levels found with the newer antibiotics agree closely with the small series described by Finland, Frank, and Wilcox (1950), except that they found that higher streptomycin concentrations were required for complete inhibition. In the series of 104 strains of staphylococci described by Spink (1951) strains very highly resistant to streptomycin were also highly resistant to penicillin, though all were sensitive to aureomycin and terramycin. Spink described in his series only one strain that was resistant to aureomycin (62.5 μg.), obtained from a child after several weeks of aureomycin treatment. In his series five strains were terramycin-resistant; in no case had the patients concerned received terramycin; the most resistant strain required 125 μg./ml. for inhibition. There were also seven chloramphenicol-resistant strains requiring 62.5 μg./ml. or more for inhibition and unrelated to previous treatment with the drug.

From this evidence it is apparent that resistance to antibiotics of a strain of staphylococcus isolated from fresh acute lesions bears little or no relation to previous antibiotic therapy given to the patient from whom the strain is obtained. The series described gives an optimistic picture of the present value of aureomycin and terramycin in treating staphylococcal infection in this country, although it is not unlikely that strains of staphylococcus resistant to them will occur with increasing frequency, especially in infections acquired in hospital where these antibiotics are widely used. Ten strains required over 4 μg. of streptomycin and 11 over 4 μg. of chloramphenicol for inhibition. As 4 μg. per ml. is a therapeutic blood level for these antibiotics, they have a place in the treatment of staphylococcal infection, though at present they appear least active, weight for weight, of the five antibiotics tested.

Summary

The sensitivity to penicillin, aureomycin, terramycin, streptomycin, and chloramphenicol of 118 strains of coagulase-positive staphylococci isolated from acute lesions in out-patients is described.

Nearly one-fifth of the strains were resistant to penicillin. Only two strains were resistant to terramycin and three to aureomycin.

In out-patients previous antibiotic therapy does not appear to influence the likelihood of encountering resistant staphylococci in acute lesions occurring later.

References

Anderson, D. G., Howard, L. G., and Rammelkamp, C. H. (1944). *Arch. Surg., Chicago*, **49**, 245.
Anderson, K. (1951). *Journal of Clinical Pathology*, **4**, 355.
Barber, M., and Rozwadowska-Dowzenko, M. (1948). *Lancet*, **2**, 641.
Blair, J. E., Carr, M., and Buchman, J. (1946). *J. Immunol.*, **52**, 281.
Demerec, M. (1945). *Proc. nat. Acad. Sci., Wash.*, **31**, 16.
Finland, M., Frank, P. F., and Wilcox, C. (1950). *Amer. J. clin. Path.*, **20**, 325.
Forbes, G. B. (1949). *Brit. med. J.*, **2**, 569.
Rountree, P. M., Barbour, R. G. H., and Thomson, E. F. (1951). *Lancet*, **1**, 435.
Spink, W. W. (1951). *J. Lab. clin. Med.*, **37**, 278.
— Ferris, V., and Vivino, J. J. (1944). *Proc. Soc. exp. Biol., N.Y.*, **55**, 210.

J. clin. Path. (1952), **5**, 169.

THE INCIDENCE OF ANTIBIOTIC RESISTANCE IN GRAM-NEGATIVE BACILLI IN A GENERAL HOSPITAL

BY

EDGAR F. THOMSON

From the Fairfax Institute of Pathology, Royal Prince Alfred Hospital, Sydney

(RECEIVED FOR PUBLICATION OCTOBER, 1951)

Five antibiotics—penicillin, streptomycin, aureomycin, chloromycetin, and terramycin—are now readily available for the treatment of diseases due to bacterial infection, and for the treatment of wounds infected either primarily or by hospital cross-infection. There is considerable overlapping in the selective activity of these antibiotics in that they are effective against Gram-positive bacteria in varying degree. The last four are also variably effective against the Gram-negative bacteria. There is, in addition, absolute selective activity in regard to some micro-organisms. Streptomycin alone is therapeutically effective against *Myco. tuberculosis*; chloromycetin has a special selective action against the *Rickettsiae*; aureomycin and terramycin have a special selective action against the larger viruses. It is, therefore, important in every case to attempt to obtain a pure culture of the infecting organism, and to evaluate its sensitivity to the various antibiotics.

The widespread use of antibiotics has also produced the problem of antibiotic resistant organisms, a problem which is as important, in relation to the Gram-negative bacilli, as it is to the Gram-positive cocci, and it was felt that an analysis of the results of routine sensitivity tests over 12 months might yield significant information.

Bact. coli, P. vulgaris, Ps. pyocyanea, H. influenzae, and Bact. friedländeri were studied with regard to their sensitivity to streptomycin, aureomycin, chloromycetin, and terramycin.

The period under review is the 12 months from January 1, 1950, to December 31, 1950. From January 1, 1950, to June 30, 1950, streptomycin only was available. From July 1, 1950, onwards aureomycin, chloromycetin, and terramycin were gradually added to the battery of antibiotics, until, in the final period, October 1, 1950, to December 31, 1950, all four antibiotics were used in the sensitivity tests.

The blood-agar plate medicated disc method of sensitivity testing has been used throughout. The method is simple and gives all the information that is required for control of antibiotic therapy. The antibiotics used in the tests were prepared in the following strengths.

Penicillin	500 units per ml.
Streptomycin	20 mg. per ml.
Aureomycin, chloromycetin, and terramycin	5 mg. per ml.

One drop of the antibiotic was placed on the paper disc. A control organism was cultured on each plate.

Results

Bact. coli.—Three hundred and twenty-four strains have been tested with streptomycin, 177 strains with aureomycin, 168 strains with chloromycetin, 137 strains with terramycin.

The 12-month period has been subdivided into (1) January 1 to June 31, 1950; (2) July 1 to September 30, 1950; (3) October 1 to December 31, 1950. The results are shown in Table I.

TABLE I

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *Bact. coli*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive		Resistant	
			No.	%	No.	%
January 1 to June 30, 1950	Streptomycin only	115	61	53·1	54	46·9
July 1 to September 30, 1950	Streptomycin	87	45	51·7	42	48·3
	Aureomycin	65	45	69·2	20	30·8
	Chloromycetin	56	45	80·3	11	19·7
	Terramycin	25	19	76·0	6	24·0
October 1 to December 31, 1950	Streptomycin	122	64	52·4	58	47·6
	Aureomycin	112	43	38·3	69	61·7
	Chloromycetin	112	76	67·0	36	33·0
	Terramycin	112	73	65·0	39	35·0
July 1 to December 31, 1950	Streptomycin	209	109	52·1	100	47·9
	Aureomycin	177	88	49·7	89	50·3
	Chloromycetin	168	121	72·0	47	28·0
	Terramycin	137	92	67·1	45	32·9

The figures show that during these three periods the percentage of strains resistant to streptomycin has remained constant at 47–48%. This stabilization might be expected, as streptomycin had been in free use within the hospital for at least 15 months before the beginning of the period under review. Observations made on *Staph. pyogenes* over the same periods of time (Rountree, Barbour, and Thomson, 1951, and unpublished data) have shown that the percentage of streptomycin-resistant strains isolated in this hospital has now become stabilized around 25% to 30%. Although there is no direct evidence, such as is available for *Staph. pyogenes*, that streptomycin-resistant strains of *Bact. coli* have arisen in, and are characteristic of, hospitals, it is not unreasonable to suppose that such is the case.

There has been an increase in aureomycin-resistant strains in the three months ending December, 1950, from 30.8% to 61.7%. This increase is statistically significant ($\chi^2 = 13.098$; $P > 0.01$). A study of the values obtained in the next three-month period should show whether this increase is of a permanent nature. A similar trend may be occurring in relation to chloromycetin-resistant and terramycin-

resistant strains. This cannot, however, be established with any certainty until at least a further three-month period has been reviewed.

Another fact which emerges from the data is the high percentage of strains sensitive to chloromycetin (72%) and to terramycin (67.1%).

On analysing the figures for the period July to December, 1950, it was noted that 26 of 137 strains (19.4%) were completely resistant to all four antibiotics.

P. vulgaris.—Two hundred and twenty-one strains have been tested with streptomycin, 77 with aureomycin and chloromycetin, and 72 with terramycin.

The 12-month period has been divided into (1) January 1 to June 30, 1950; (2) July 1 to December 31, 1950. The results are shown in Table II.

TABLE II

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *P. vulgaris*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive		Resistant	
			No.	%	No.	%
January 1 to June 30, 1950	Streptomycin only	70	61	87·1	9	12·9
July 1 to December 31, 1950	Streptomycin	151	113	74·8	38	25·2
	Aureomycin	77	6	7·8	71	92·2
	Chloromycetin	77	49	63·7	28	36·3
	Terramycin	72	11	15·2	61	84·8

The increase in streptomycin-resistant strains from 12.9% to 25.2% over these periods is barely significant ($\chi^2 = 4.17$; $P = 0.15$); 63.7% of the strains were sensitive to chloromycetin, while only 7.8% were sensitive to aureomycin, and 11% to terramycin. It is obvious that in the treatment of infections with *P. vulgaris* streptomycin and chloromycetin are the antibiotics of choice.

Ps. pyocyanea.—One hundred and thirty-one strains have been tested with streptomycin, 46 with aureomycin and chloromycetin, and 38 with terramycin.

The 12-month period has been divided into (1) January 1 to June 30, 1950; (2) July 1 to December 31, 1950. The results are shown in Table III.

TABLE III

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *Ps. pyocyanea*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive		Resistant	
			No.	%	No.	%
January 1 to June 30, 1950	Streptomycin only	68	34	50·0	34	50·0
July 1 to December 31, 1950	Streptomycin	63	26	41·3	37	58·7
	Aureomycin	46	3	6·5	43	93·5
	Chloromycetin	46	8	17·4	38	82·6
	Terramycin	38	18	47·4	20	52·6

The incidence of streptomycin-resistant strains (50–58%) has not altered significantly during the 12 months. The figures show clearly that this organism is very resistant to all antibiotic therapy, although response to terramycin may be expected in approximately 50% of cases. Aureomycin and chloromycetin would appear to be ineffective, since 80% to 90% of the strains tested were resistant to these substances.

Bact. friedländeri.—The figures shown in Table IV are for the whole period of 12 months. No percentages are given because the total number of strains investigated is too small. It should be noted, however, that the four strains investigated were all resistant to the action of terramycin, in contrast to the findings of Linsell and Fletcher (1950).

TABLE IV

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *Bact. friedländeri*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive	Resistant
January 1 to December 31, 1950	Streptomycin	35	32	3
	Aureomycin	4	3	1
	Chloromycetin	4	4	0
	Terramycin	4	0	4

H. influenzae.—The figures shown in Table V are for the whole period of 12 months, and again, because of the small numbers of strains investigated, no percentages are expressed.

TABLE V

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *H. influenzae*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive	Resistant
January 1 to December 31, 1950	Streptomycin	19	19	0
	Aureomycin	3	2	1
	Chloromycetin	3	3	0
	Terramycin	3	3	0

Streptomycin still appears to be the drug of choice for infections with *H. influenzae* and *Bact. friedländeri.*

Summary

Five micro-organisms—*Bact. coli, P. vulgaris, Ps. pyocyanea, H. influenzae,* and *Bact. friedländeri*—have been tested for sensitivity to streptomycin, aureomycin, chloromycetin, and terramycin.

The percentages of sensitive and resistant strains isolated over the 12-month period, January 1, 1950, to December 31, 1950, are given.

Three hundred and twenty-four strains of *Bact. coli* were examined. The percentage of strains resistant to streptomycin has remained constant over the period at 47% to 48%. The percentage of strains resistant to aureomycin has increased from 30.8% to 61.7%. The increase is statistically significant ($\chi^2=13.098$; $P>0.01$). A similar trend may be occurring in relation to the chloromycetin and terramycin.

Two hundred and twenty-one strains of *P. vulgaris* were tested with streptomycin ; 77 with aureomycin and chloromycetin, 72 with terramycin. There has been an increase in the streptomycin-resistant strains from 12.9% to 25.2% over the period. This is barely statistically significant ($\chi^2=4.17$; $P>0.15$). Of the strains tested with chloromycetin, 63.7% were sensitive. Streptomycin and chloromycetin are the antibiotics of choice in infections with *P. vulgaris*.

One hundred and thirty-one strains of *Ps. pyocyanea* have been tested with streptomycin ; 46 with aureomycin and chloromycetin, 38 with terramycin. This organism is very resistant to all antibiotic therapy, but response to terramycin and streptomycin may be expected in approximately 50% of cases.

A small series of results with *Bact. friedländeri* and *H. influenzae* is shown. The four strains of *Bact. friedländeri* tested were all resistant to terramycin. Streptomycin still appears to be the antibiotic of choice for infections with these two organisms.

I should like to thank Dr. Phyllis Rountree for her valuable help and criticism, and Mr. George Barr and Miss Vera Sife for their technical assistance.

REFERENCES

Linsell, W. D., and Fletcher, A. P. (1950). *Brit. med. J.*, **2**, 1190.
Rountree, P. M., Barbour, R. G. H., and Thomson, E. F. (1951). *Lancet*, **1**, 435.

ADDENDUM

Since this paper was written further tests have been carried out in the period January 1, 1951, to March 31, 1951, and the results are shown in Tables VI, VII, and VIII. During this period 514 strains of *Bact. coli*, 160 strains of *P. vulgaris*, and 83 strains of *Ps. pyocyanea* were tested. When the results are compared with those for the period July 1, 1950, to December 31, 1950, it will be seen that the

TABLE VI

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *Bact. coli*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive		Resistant	
			No.	%	No.	%
January 1 to March 31, 1951	Streptomycin	514	248	48·2	266	51·8
	Aureomycin	514	166	32·2	348	67·8
	Chloromycetin	514	300	58·4	214	41·6
	Terramycin	514	319	62·2	195	37·8

TABLE VII

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN, AND TERRAMYCIN OF STRAINS OF *P. vulgaris*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive		Resistant	
			No.	%	No.	%
January 1 to March 31, 1951	Streptomycin	160	108	67·5	52	32·5
	Aureomycin	160	20	12·5	140	87·5
	Chloromycetin	160	101	63·1	59	36·9
	Terramycin	160	21	13·1	139	86·9

TABLE VIII

SENSITIVITY TO STREPTOMYCIN, AUREOMYCIN, CHLOROMYCETIN AND TERRAMYCIN OF STRAINS OF *Ps. pyocyanea*

Period of Isolation	Antibiotic	Total No. of Strains	Sensitive		Resistant	
			No.	%	No.	%
January 1 to March 31, 1951	Streptomycin	83	39	47·0	44	53·0
	Aureomycin	83	6	7·2	77	92·8
	Chloromycetin	83	14	16·9	69	83·1
	Terramycin	83	24	29·0	59	71·0

trend is towards an increase in resistant strains, e.g. the strains of *Bact. coli* resistant to aureomycin have risen from 50.3% to 67.8% ; those resistant to chloromycetin have increased from 28.0% to 41.6% ; the strains of *Ps. pyocyanea* resistant to terramycin have increased from 52.6% to 71.0%.

J. clin. Path. (1952), **5**, 175.

CORONARY ARTERIOSCLEROSIS AND CALCIFICATION IN INFANCY

BY

R. A. SLADDEN

From the Department of Morbid Anatomy, Radcliffe Infirmary, Oxford

(RECEIVED FOR PUBLICATION SEPTEMBER 27, 1951)

Several reports have appeared in recent years in the American literature dealing with the occurrence of arteriosclerosis and arterial calcification in infancy. This process may affect the coronary arteries in particular, possibly giving rise to myocardial infarction.

Although the condition is mentioned briefly in some textbooks of pathology and paediatrics, it has attracted few reports from this country. The latest of these appears to be that of Lightwood (1932), if that case can be said to have occurred during infancy, the child having died at 27 months. It is probable that some isolated cases have not been reported.

Field (1946) and Stryker (1946) both indexed the available cases up to that time, including Lightwood's case, added personally studied cases, and gave comprehensive reviews on the question of aetiology. Out of a total of 25 cases of arterial calcification referred to by these authors, 22 showed involvement of the coronary arteries. A similar type of case was reported by Andersen in 1945. A year later Lingmark and Murray added a further case, and referred to another case in the literature, that of Scheidegger (1940). Cases have since been reported by Hause and Antell (1947, one case); Prior and Bergstrom (1948, two cases); Menten and Fetterman (1948, three cases). Brief reference was made by Weller (1940) to an apparently similar type of case. Those few reports in the literature which describe arterial calcification in infancy, without the mention of coronary arterial involvement, are not considered here.

It appears to be generally agreed that no single aetiological factor has been identified as the cause of this condition. There is evidence in some cases that renal disease may play an important role; arterial calcification is known to have occurred in some cases of renal rickets in older children (Shelling and Remsen, 1935; Smyth and Goldman, 1934). Even if such cases as those with gross renal pathology (Bryant and White, 1901; Andersen and Schlesinger, 1942; Lightwood, 1932; Stryker, Case 4, 1946) are excluded from consideration, there still remains a majority for which no satisfactory aetiology has been found. To this latter group belong the two cases reported below.

Other reputed aetiological factors include syphilis, other infections, allergy, and hypervitaminosis D.

Clinical Features

The disease has been reported in a stillborn infant (Stryker, 1946) and in a child of 27 months (Lightwood, 1932).

There are 19 males, 12 females, and two cases of unstated sex in a total of 33 cases, including the two cases reported here.

The family history may be of importance, since Menten and Fetterman (1948) reported the disease in two siblings.

There have been no constant findings of apparent significance in the histories of pregnancy, delivery, or upbringing. Although the administration of vitamin D or calcium supplements to mother or child is referred to in some reports, in only one case do the authors feel justified in incriminating hypervitaminosis D as an aetiological factor (Ross and Williams, 1939).

Once the child becomes ill there is usually a fairly steady downhill course terminating in death in a few days or weeks. Sudden death has been reported. The child refuses its feeds, loses weight, and may begin to vomit. Clinical signs are usually referred to the cardiovascular or respiratory systems, but may be completely absent. Cardiac enlargement, periodic attacks of cyanosis and dyspnoea, enlargement of the liver and spleen, ankle oedema, and ascites may be found. Congestion of the lungs associated with low-grade pyrexia sometimes suggests a diagnosis of bronchopneumonia, which, in fact, often supervenes terminally. Diarrhoea and other terminal infections have also been reported. Bryant and White (1901) reported gangrene of the right foot as a result of arterial occlusion. In the second case reported below it is almost certain that the child was suffering from paroxysmal attacks of angina pectoris. In Case 1 of Prior and Bergstrom (1948) involvement of the meningeal and cerebral vessels gave rise to neurological signs. Radiographs of the limbs in this case, taken during life, were re-examined after death and showed calcification of the limb vessels, but radiological and laboratory investigations are usually of little value. The highest value obtained for serum calcium was in Case 1 of Menten and Fetterman (1948), the level at necropsy being 16.5 mg./100 ml. The serum phosphates were 4.86 mg./100 ml.

Pathological Features

The essential pathological lesion is found in the medium-sized arteries of the body, including the coronary arteries, and consists of fibroblastic proliferation of the intima with calcification occurring in close relationship to the internal elastic lamina and spreading into the medial coat and proliferated intima. There are varying degrees of disorganization of the vessels, obstruction of the lumina and recanalization, round cell, and occasional giant cell or eosinophilic infiltration of the vessel walls. These changes have been reported from nearly all parts of the body, including the brain, lungs, abdominal viscera, and limbs. The aorta may also show some calcification in relation to the elastic fibres of the media. If coronary occlusion occurs, myocardial necrosis and calcification of the affected muscle may take place. The left ventricle and papillary muscles are frequently involved.

Various types of pneumonia have been described in this series.

Parathyroid hyperplasia was reported by Andersen and Schlesinger (1942), probably secondary to bilateral hydronephrosis and congenital hypoplastic kidneys in one case, and polycystic kidneys in the other. Stryker (1946) reported polycystic

kidneys and multiple congenital abnormalities in his case 4; Bryant and White (1901) recorded congenital urethral dilatation with hydronephrosis, and in Oppenheimer's (1938) Case 2 there was a congenital malformation of the genitalia.

Early or chronic glomerulo-nephritis has been reported in a few cases, and Lightwood (1932) described glomerular and interstitial fibrosis with extensive calcinosis of the kidney in his case of dwarfism.

Andersen (1945) observed extensive aortic involvement associated with cirrhosis and interstitial calcification of the liver. The infant died shortly after birth: no evidence of syphilis was obtained.

Case Reports

Case 1 (Reg. No. R.I. 5254/39).—A boy, aged 18 months, was born normally, three weeks prematurely. He was breast-fed for one year, then weaned on half a cup of cow's milk a day.

Neither mother nor child received vitamin or calcium supplements.

At the age of 14 months he fell and hurt his head, afterwards developing a sore throat and anorexia. Nine weeks later he was admitted to hospital.

On admission he was seen to be a pale, lethargic infant with slight cyanosis of the lips. The heart was found to be enlarged on clinical examination. The liver was palpable one fingerbreadth below the costal margin. One week later the feet became oedematous and remained thus for three days. Numerous intermittent attacks of fever and dyspnoea with some liver enlargement occurred before death, which took place in a final attack. There had been some vomiting and progressive emaciation. The clinical diagnosis was pneumonia.

Post-mortem Examination (P.M. 425/39).—The body was that of an emaciated male infant.

Lungs.—There were signs of chronic congestion, and the lungs were rather indurated.

Heart.—The heart weighed 137 g., and there was a sterile, gelatinous pericardial exudate. Generalized cardiac hypertrophy, especially of the left ventricle, was seen and endocardial thickening, especially in the region of the mitral valve.

Liver.—The liver was enlarged, firm and congested.

Kidneys.—The kidneys were congested, with several recent haemorrhagic cortical infarcts and adherent capsules.

Suprarenal.—Marked congestion in the deep cortical layers was seen.

Histological Examination.—The following organs were examined histologically.

Lungs.—There was a considerable degree of vascular congestion together with early bronchopneumonic changes. Many alveolar macrophages contained vacuoles suggestive of inhalation lipoid pneumonia.

Heart.—Some hypertrophy of myocardial fibres was seen. A branch of the coronary artery showed calcification in relation to the internal elastic lamina and slight irregular intimal proliferation.

Liver.—In the liver there was considerable fatty change, centrilobular congestion and necrosis. Occasional round-cell infiltration of the portal tracts was seen.

Kidneys.—These showed areas of calcification involving the tubules and main renal arterial vessels. Small focal areas of calcification of tubular epithelium were seen, with occasional extrusion of the calcified masses into the lumina of the tubules.

The affected tubules were usually in close relation to glomeruli and were probably of the proximal convoluted type. Very occasional foci of calcification were seen within

glomeruli of a rather immature type; the more mature glomeruli were apparently not affected.

Many of the main branches of the renal artery showed irregular plaques of intimal proliferation and deposition of calcium along the underlying internal elastic laminae. These deposits quite often extended into the media forming roughly spherical masses, but there did not appear to be any medial hypertrophy or inflammatory reaction either of the media or intima.

Linear deposits of calcium were occasionally found along the internal elastic laminae of some of the smaller branches of the renal arteries, but arterioles were not affected and the vascular changes were confined to the medulla and pelvic tissues, whereas focal tubular calcification was scattered throughout the renal parenchyma.

Pancreas.—Large arteries in the region of the pancreas showed changes similar to those seen in the renal arteries.

Suprarenals.—Arterial calcification of the type already described was found in the suprarenal arteries, but more striking changes were found in the sinusoidal vessels of the zona reticularis and medulla. The majority of these contained irregular calcified bodies of varying sizes, often laminated, and sometimes containing at one edge a cell nucleus suggestive of an endothelial origin. It appeared that the calcium might have been deposited directly upon, or within, the endothelial lining cells.

The cells of the suprarenal cortex and medulla showed no abnormalities.

The pathological diagnosis was calcification of the coronary arteries and medium-sized arteries of the tongue, pancreas, and kidneys; calcification of the sinusoids of the suprarenal medulla; myocardial hypertrophy; low-grade bronchopneumonia; acute pericarditis.

Case 2* (Reg. No. R.I. 129655).—A boy, aged 3 months, was born normally after a full term pregnancy. He was breast-fed for three weeks, then given national dried milk and the usual cod-liver oil supplements. Weight was gained steadily. At the age of three months he refused his feed one evening and the same night began crying. Twenty hours later respirations became rapid and gasping. He had two further attacks of dyspnoea, screaming, and evident pain which appeared to be abdominal in origin. The attacks were followed by collapse and cyanosis, the extremities becoming cold and clammy.

On admission to hospital the liver and spleen were both palpable. No pus cells or red cells were found in the urine. Radiographs of the chest and abdomen and a barium enema showed no definite lesion.

In view of the severe pain, presumably of abdominal origin, laparotomy was performed but no abnormality was found. Four hours after operation and two days after the beginning of the illness the child suddenly collapsed and died.

The parents were both alive and well. There was one healthy sibling aged 3 years. The mother had not received vitamin D therapy.

Post-mortem Examination (P.M. 391/50).—The body was that of a normally developed male child.

Lungs.—Patchy areas of atelectasis were seen.

Liver and Spleen.—The liver and spleen were congested.

Arteries.—The mesenteric, femoral, and tibial arteries were calcified; numerous arteries in the region of the thyroid gland were enlarged, calcified, and tortuous.

---

* The radiological aspects of this case have been reported briefly by Boldero (1951).

Heart.—The heart, together with the aortic arch and the roots of the great vessels, weighed 60 g. The heart measured 7 × 4.5 × 4.3 cm. and the left ventricular wall measured from 0.8 to 1.3 cm. in thickness. The coronary arteries were conspicuous, hard and tortuous (Fig. 1), and there was a small subepicardial haemorrhage around the circumflex branch of the left coronary artery. There was necrosis of the anterior papillary muscle of the mitral valve and of a small area of the interventricular septum. Radiography of the viscera revealed calcification of the hepatic, pulmonary, splenic, pancreatic, renal, and suprarenal arteries.

The majority of the calcified vessels showed irregular thickening with a somewhat beaded appearance.

The parathyroid glands could not be identified on naked-eye examination. Other organs appeared natural apart from the changes mentioned above. Blood was taken for serum calcium estimation and Wassermann and Kahn reactions.

Histological Examination.—Blocks of tissue were taken from the principal viscera, and also from the carotid, femoral, tibial, brachial, and mesenteric arteries. Fixation was carried out in Gros and Lehan's buffer fixative and in chilled acetone.

All blocks were stained by haematoxylin and eosin ; many by orcein and toluidine blue for elastica and the periodic-acid-Schiff method for polysaccharides.

Gomöri's alkaline phosphatase stain and Von Kossa's stain for calcium were applied to several blocks of the arterial tissue.

Lungs.—Early bronchopneumonic changes were seen. One of the main branches of the pulmonary artery showed scattered linear zones of calcification along the elastic fibres of the media. In general the intima was normal in appearance, but in one area there was some subendothelial fibroblastic proliferation and underlying calcification of the internal elastic lamina.

Heart.—The intima of the coronary arteries (Fig. 2) was generally thickened by subendothelial fibroblastic proliferation, while a few small elastic fibres were seen in its deeper layers. Calcification was seen along the internal elastic lamina which in places was interrupted by a larger plaque of calcification. Spicules of calcium extended radially into the intima. The media, which was relatively thin, showed some diffuse deposition of calcium. As a result of the intimal thickening the lumen of the affected artery was considerably reduced. The myocardium of the left ventricle showed some hypertrophy, and in the interventricular septum and papillary muscles areas of ischaemic necrosis and calcification, surrounded by diffuse fibrosis and some inflammatory reaction (Fig. 4), were seen. The inflammatory cells were chiefly polymorphonuclear leucocytes, including a moderate number of eosinophils.

Liver.—A slight degree of centrilobular congestion was present. The hepatic cells showed no significant changes. There was some periportal acute inflammatory cell infiltration. The larger branches of the hepatic artery were the only ones apparently affected by the process of calcification.

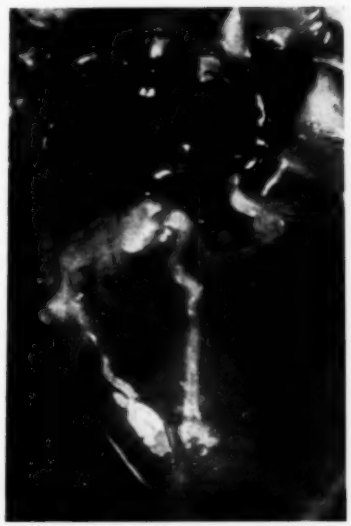


FIG. 1.—Case 2. Heart, showing prominent and tortuous branches of the right coronary artery.

Spleen.—The spleen was congested, with prominent follicles and a moderately cellular pulp. A fairly small branch of the splenic artery showed focal calcification of the media without intimal thickening.

Kidneys.—With the exception of a very occasional focus of calcification of tubular epithelium, the renal parenchyma exhibited no significant pathological changes. Many of the larger branches of the renal arteries, however, showed focal areas of calcification of the media and internal elastic lamina with some overlying intimal proliferation and occasional calcification of the proliferated intima itself.

Suprarenals.—The cortex and medulla showed no pathological changes and no calcification of the medullary sinusoids was seen.

One of the suprarenal arteries (Fig. 3) showed almost complete obliteration of the lumen by intimal proliferation and calcification. Two multinucleate giant cells were seen at the periphery of the calcified zone. The media appeared to have escaped calcification, but both the media and the intima showed a moderately intense infiltration by eosinophil leucocytes.

Aorta.—An occasional small area of calcification was found in the medial coat. The intima was somewhat irregular in thickness and at one or two points there was fragmentation of the internal elastic lamina with very early deposition of calcium.

Other Blood Vessels.—Changes similar to those already described were found in the femoral, tibial, and mesenteric arteries, and in the arteries of the thyroid region. Some round cell infiltration surrounded the plaques in the femoral artery and a few foreign-body giant cells were also seen.

Calcification was very marked in the thyroid arteries, but was nowhere as advanced as that seen in the suprarenal artery.

Other Organs.—The remaining viscera, the brain and cerebral vessels, and the femoral and sternal bone marrow showed no significant pathological changes. The parathyroid glands were not identified microscopically.

The presence of calcium was confirmed by the use of Von Kossa's stain. It was not possible to demonstrate the presence of phosphatases in the arterial walls by the use of Gomöri's stain.

The serum calcium was 8.9 mg./100 ml. The Wassermann and Kahn reactions were negative.

The pathological diagnosis was calcification of the pulmonary, coronary, hepatic, splenic, mesenteric, pancreatic, renal, suprarenal, thyroid, femoral, and tibial arteries, and of the aorta ; myocardial infarction with calcification ; occasional calcification of renal tubular epithelium ; early bronchopneumonia.

Discussion

The intimal changes seen are presumably secondary to the calcification so that from a morphological standpoint this process of calcification is typically metastatic, there being no evidence of pre-existing arterial disease. The rapidity of calcification of the ischaemic myocardium, however, suggests an unusual facility for dystrophic calcification. If the current theories of aetiology, namely, either a disturbance of the ground-substance of the arterial walls or a defect in the elastica, have any basis, then the calcification may be regarded as dystrophic and failure to demonstrate known causes of metastatic calcification is not surprising. These causes in man have been listed by Mulligan (1947) as bone disease, primary parathyroid neoplasm, chronic renal disease, and hypervitaminosis D.

The occurrence of the disease in siblings, reported by Menten and Fetterman (1948), together with some other cases suggestive of a familial incidence, might

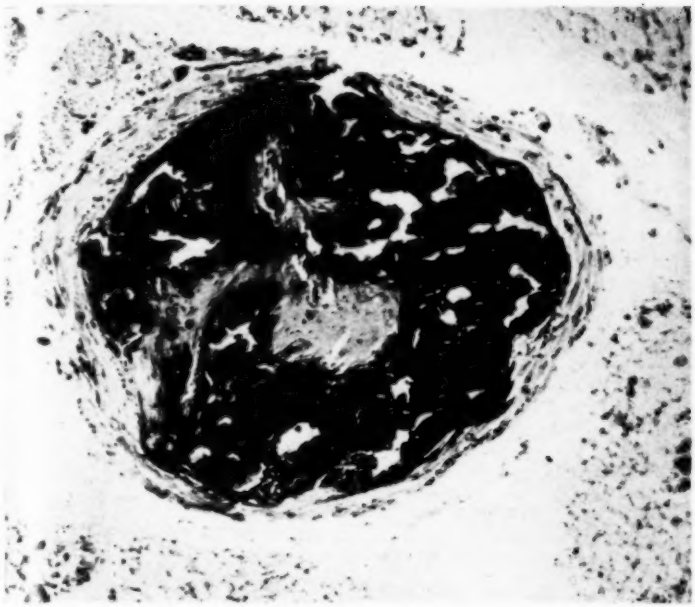


FIG. 3

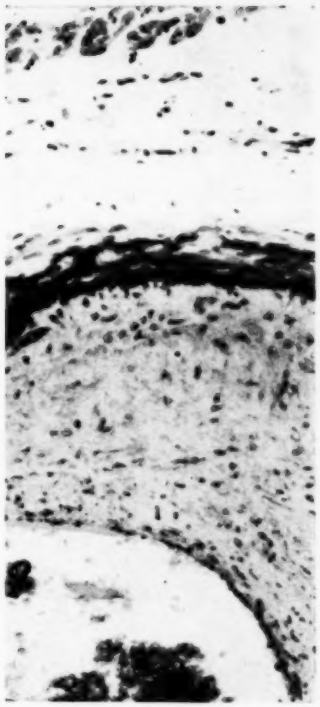


FIG. 2

FIG. 2.—Case 2. Section through left coronary artery near its origin, showing fibroblastic proliferation of the intima and calcification around the internal elastic lamina. (H. and E.) × 170.

FIG. 3.—Case 2. Section of suprarenal artery and surrounding fatty tissue, showing almost complete obliteration of the arterial lumen by intimal proliferation and calcification. (H. and E.) × 85.

FIG. 4.—Case 2. Section of papillary muscle of heart, showing myocardial necrosis and calcification. (H. and E.) × 20.

FIG. 4

favour the theory of some inherent biochemical aberration in ground-substance or elastica. Certainly the process of calcification appears to be closely related to the elastica of the arterial walls.

Minkowski (1947) has studied the formation of intimal cushions and the elastic hyperplasia which not infrequently occurs in the coronary arteries of infants, regarding infection as an important factor. He did not observe calcification in the coronary arteries, but the lability of the arteries in early infancy may play some part in the pathogenesis of the condition under discussion.

As noted by Stryker (1946), the similarity of this condition to the calcification of arteries seen occasionally in young subjects is also of interest. The latter process is seen sometimes in arteries of the thyroid region in otherwise healthy individuals. In Case 2 described above calcification in this area was very marked.

Increased recognition of this disorder may perhaps enable the clinical or radiological diagnosis to be made, and thereby lead to fuller biochemical investigations during life and to prompt anatomical and histochemical studies.

I wish to thank Dr. A. H. T. Robb-Smith for the valuable advice and assistance he has given, and for permission to use the post-mortem report on the first case ; Dr. V. Smallpeice for permission to publish both cases ; and Mr. R. G. Hill for the photography.

References

Andersen, D. H., and Schlesinger, E. R. (1942). *Amer. J. Dis. Child.*, **63**, 102.
Andersen, S. R. (1945). *Acta path. microbiol. scand.*, **22**, 180.
Boldero, J. L. (1951). *Brit. J. Radiol.*, **24**, 43.
Bryant, J. H., and White, W. H. (1901). *Guy's Hosp. Rep.*, **55**, 17.
Field, M. H. (1946). *Arch. Path., Chicago*, **42**, 607.
Hause, W. A., and Antell, G. J. (1947). Ibid., **44**, 82. (Corr. p. 438).
Lightwood, R. (1932). *Arch. Dis. Childh.*, **7**, 193.
Lingmark, I., and Murray, U. (1946). *Nord. Med.*, **29**, 183.
Menten, M. L., and Fetterman, G. H. (1948). *Amer. J. clin. Path.*, **18**, 805.
Minkowski, W. L. (1947). *Amer. J. med. Sci.*, **214**, 623.
Mulligan, R. M. (1947). *Arch. Path., Chicago*, **43**, 177.
Oppenheimer, E. H. (1938). *Johns Hopk. Hosp. Bull.*, **63**, 261.
Prior, J. T., and Bergstrom, V. W. (1948). *Amer. J. Dis. Child.*, **76**, 91.
Ross, S. G., and Williams, W. E. (1939). Ibid., **58**, 1142.
Scheidegger, S. (1940). *Frankf. Z. Path.*, **54**, 442. Cited by Lingmark and Murray.
Shelling, D. H., and Remsen, D. (1935). *Johns Hopk. Hosp. Bull.*, **57**, 158.
Smyth, F. S., and Goldman, L. (1934). *Amer. J. Dis. Child.*, **48**, 596.
Stryker, W. A. (1946). *Amer. J. Path.*, **22**, 1007.
Weller, C. V. (1940). Ibid., **16**, 666.

J. clin. Path. (1952), **5**, 183.

THE DIAGNOSIS OF RETICULOSARCOMA OF THE THYROID GLAND

BY

G. B. D. SCOTT

From the Department of Pathology, University of Aberdeen

(RECEIVED FOR PUBLICATION OCTOBER 16, 1951)

The difficulty of making a histological distinction between reticulosarcomata and certain anaplastic carcinomata is well known and this applies particularly to the thyroid gland. From a study of the literature it is not easy to obtain an accurate idea of the frequency of reticulosarcomata of the thyroid, using the term reticulosarcoma to include lymphoblastic and lymphocytic (lymphosarcoma) tumours as well as the reticulum cell and other varieties (Robb-Smith, 1938).

Rice (1932) described four cases of lymphoblastic reticulosarcoma of the thyroid and one of a more primitive type, possibly a reticulum cell tumour, these cases occurring between the years 1922 and 1929. Vaux (1937) described a case of dictyocytic reticulosarcoma of the thyroid occurring amongst 25 malignant thyroid tumours. Portmann (1940), in a review of 184 malignant thyroid tumours, recorded the presence of 11 cases of lymphosarcoma, but did not subdivide them into their predominant cell types. He also recorded 14 cases as " unclassifiable," but did not state why this was so or give any description of these tumours. Joll (1941) reviewed 127 malignant thyroid tumours and recorded seven cases of sarcoma. He stated that the reticulum cell variety was the commonest, but gave no figures. The Massachusetts General Hospital recorded, with necropsy findings, four examples of reticulosarcoma of the kidneys (1939, 1941, 1948), one of which (Case No. 34032, 1948) showed coexistent reticulosarcoma of the thyroid. Kellett and Sutherland (1949) recorded five cases of thyroid reticulosarcoma. From the description given of them, one appeared to be of polymorphic type, two reticulum cell, and two lymphoblastic. However, they did not state over what period of time these tumours occurred nor what percentage they formed of the total malignant thyroid tumours encountered in that period. Of the 43 primary malignant thyroid tumours examined histologically in this department between January 1, 1938, and January 1, 1951, 13 were predominantly anaplastic but with small foci of obvious carcinoma, while nine were completely anaplastic, and, of these, only one (Case 3 below) was proved by subsequent events to be a lymphoblastic reticulosarcoma.

There can be no doubt that the frequency with which a diagnosis of reticulosarcoma of the thyroid is made varies greatly according to the criteria acceptable to different observers, and it is with the intention of evaluating these diagnostic criteria that the following case of anaplastic carcinoma and two cases of authenticated reticulosarcoma of the thyroid gland are presented.

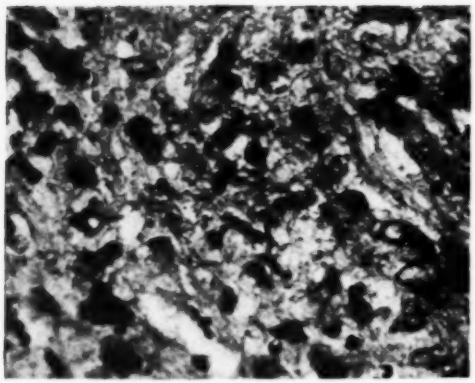


FIG. 1.—Anaplastic thyroid tumour in Case 1. Haematoxylin and eosin. × 700.

FIG. 2.—Anaplastic thyroid tumour in Case 1, showing reticulin network. Silver impregnation. × 700.

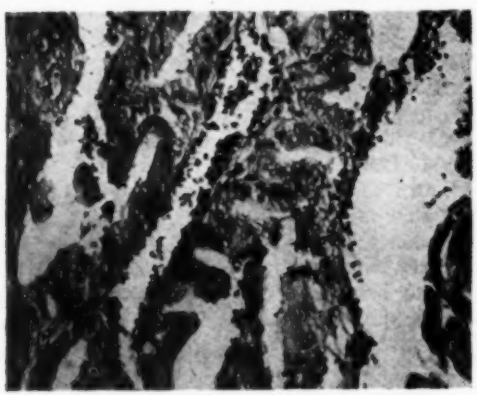


FIG. 3.—Area of papillary carcinoma in Case 1. Haematoxylin and eosin. × 180.

Case Histories

Case 1.—J. K., a man aged 49, was admitted to Woodend Hospital, Aberdeen, on June 2, 1950, complaining of pain in the left side of the face and neck and of dysphagia of four months' duration. On June 7 he was operated upon for the removal of a retrosternal mass, which proved to be a malignant tumour, partially necrotic, arising from the left lobe of the thyroid gland and adherent to the great vessels, trachea, and oesophagus. Only part of the mass could be removed and *x*-ray therapy was begun. He subsequently developed radiological evidence of metastases in both lungs, pelvis, and right femur. He died on November 11, 1950. No necropsy was performed.

Histology.—In general, the tumour was anaplastic and highly cellular with some pleomorphism (Fig. 1). The predominant cell possessed a large oval nucleus with a prominent nucleolus, not unlike a reticulum cell. Also, silver impregnation revealed a fine supporting reticulin network (Fig. 2) in intimate relationship to the anaplastic cells, although numerous fragmented strands of pre-existing reticulin could be identified. Other small areas, however, presented the unmistakable features of a carcinoma, evidenced by cuboidal cells and a papillary structure (Fig. 3).

The conjunction of neoplastic cells resembling reticulum cells and a fine intercellular reticulin network might well have led to an erroneous diagnosis of reticulosarcoma, had not the presence of a relatively inconspicuous carcinomatous foci indicated beyond all doubt the true nature of the thyroid tumour.

Case 2.—E. A., a married woman aged 63, was admitted to the Broadstone Jubilee Hospital, Port Glasgow, on January 1, 1949, complaining of a swelling of the thyroid gland which had been present for three months without producing any respiratory distress. Examination revealed that, although the

whole thyroid gland was affected, the enlargement was most marked in the right lobe, which was hard and fixed to the deep tissues. A clinical diagnosis of a malignant thyroid tumour was made and partial removal of the right lobe was carried out, no enlarged lymph nodes being encountered. The post-operative course was uneventful and *x*-ray therapy was subsequently given.

She was admitted to the Aberdeen Royal Infirmary on July 8, 1949, having suffered from intermittent haematuria for approximately four and a half months, associated with a dull ache in the lumbar region and clot colic. Investigation indicated the presence of a renal tumour on the right side. An enlarged and hard right kidney was removed. *X*-ray examination failed to reveal any signs of tumour deposits in the lungs, humeri, femora, or vertebrae.

She was readmitted to the Broadstone Jubilee Hospital on September 23, because of persistent vomiting of four weeks' duration. A barium meal showed the presence of a duodenal ulcer, but there was no clinical evidence of either a local recurrence of the thyroid tumour or of metastases. She died on October 17, 1949, no necropsy being performed.

Thyroid.—The specimen from the thyroid consisted of 42 g. of firm pale tissue.

The lesion was a highly cellular and anaplastic tumour showing considerable variation in cell size and nuclear density (Figs. 4 and 5). Numerous cells of reticulum cell type were seen, some binucleate, and mitoses were plentiful. Although considerable coarse, preformed reticulin was present, there was also much that was apparently of recent origin and in close apposition to single cells or small groups of cells (Fig. 6). In addition, the tumour was intersected by ill-defined strands of fibrous tissue.

Right Kidney.—The organ, which was uniformly enlarged and measured 14 cm. from pole to pole, was bisected vertically from the convex border to the

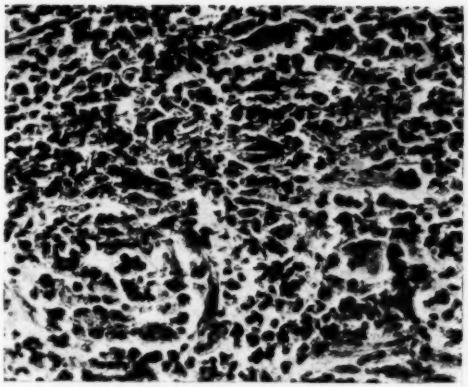


FIG. 4.—Anaplastic thyroid tumour in Case 2. Haematoxylin and eosin. × 240.

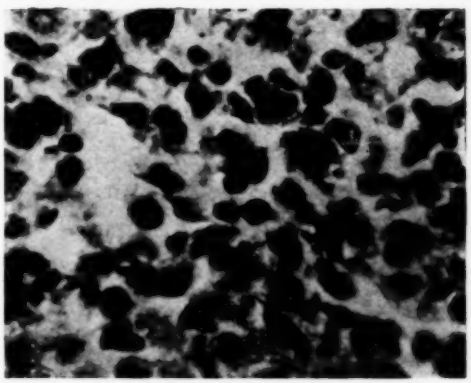


FIG. 5.—Anaplastic thyroid tumour in Case 2. Haematoxylin and eosin. × 700.

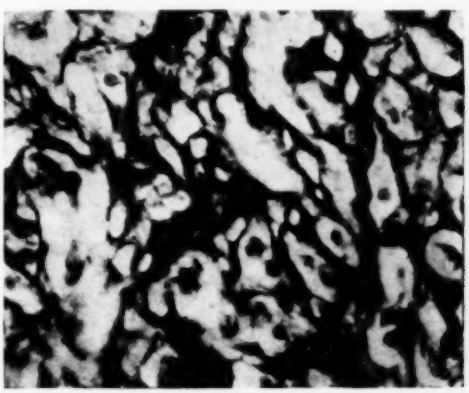


FIG. 6.—Anaplastic thyroid tumour in Case 2, showing reticulin network. Silver impregnation. × 700.

FIG. 7.—Renal tumour in Case 2, showing infiltration of tumour tissue with surviving islets of renal medulla. Measurements in centimetres (15 × 9 cm.)

hilum. Apart from small scattered areas of surviving renal medulla, the entire renal parenchyma had been replaced by a pale homogeneous tissue. There was no obvious penetration of the renal capsule and the calyces were flattened (Fig. 7).

The appearances were essentially the same as those of the thyroid tumour, as regards both the cellular elements and the reticulin network (Figs. 8, 9, and 10), and numbers of surviving glomeruli, some of them sclerosed, as well as renal tubules were present within the tumour tissue (Fig. 11).

The peculiar diffuse character of the renal tumour is similar to the renal lesions described in Case No. 34032, recorded by the Massachusetts General Hospital. It is quite unlike the usual circumscribed form of either a primary or metastatic growth and resembles the type of lesion seen in some cases of leukaemia. This, together with its histological appearance, indicates that it is a reticulosarcoma, while the histological similarity between it and the thyroid tumour shows that the latter is also a reticulosarcoma. It is impossible, however, to determine which, if either, is the primary focus, or whether the lesion is of multifocal origin.

Case 3.—M. C., a married woman aged 61, was admitted to Stracathro Hospital, Brechin, on July 21, 1947. About four weeks previously she had noticed a swelling in the region of the thyroid gland and at the same time her voice had become husky. An acute attack of dyspnoea occurred on the evening before her admission to hospital.

On examination, she was seen to be dyspnoeic and there was a slight tremor of the outstretched fingers but no exophthalmos; her pulse rate was 96 per minute. There was a diffuse enlargement of the thyroid gland, affecting the right lobe more than the left.

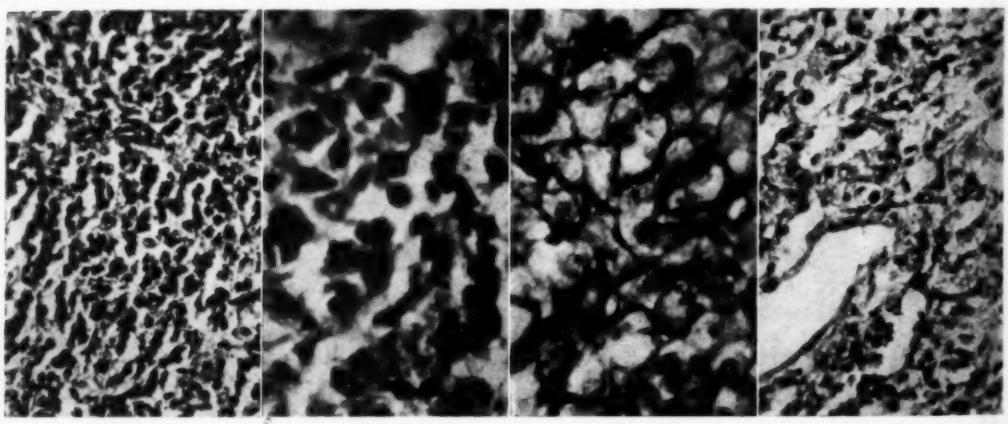


FIG. 8 FIG. 9 FIG. 10 FIG. 11

FIGS. 8, 9, 10, and 11.—Renal tumours in Case 2: (8) Showing anaplastic appearance. Haematoxylin and eosin. × 240. (9) Haematoxylin and eosin. × 700. (10) Showing reticulin network, silver impregnation. × 700. (11) Showing surviving renal tubules. Haematoxylin and eosin. × 240.

FIG. 12.—Ulcer of stomach in Case 3. Haematoxylin and eosin. × 2.

FIG. 13.—Anaplastic thyroid tumour in Case 3. Haematoxylin and eosin. × 240.

FIG. 14.—Anaplastic thyroid tumour in Case 3. Haematoxylin and eosin. × 700.

FIG. 12

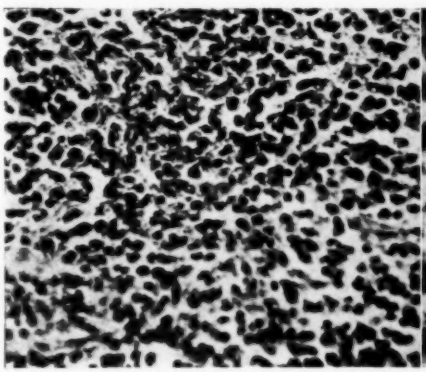


FIG. 13

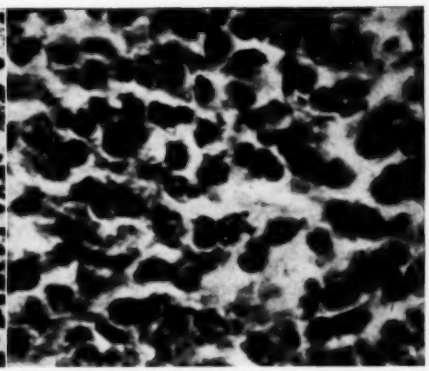


FIG. 14

The gland extended downwards behind the suprasternal notch and the trachea was displaced to the left. There was no enlargement of the regional lymph nodes. She was transferred to the Aberdeen Royal Infirmary, where, on July 26, a partial thyroidectomy was performed, when it was found that the enlarged right lobe extended behind the trachea and oesophagus, being adherent to the latter. The thyroid tissue was pale and friable and appeared to invade the surrounding muscles. The immediate post-operative course was uneventful and *x*-ray therapy to the region of the thyroid was begun. She was readmitted to Stracathro Hospital on September 24 because of an erythematous rash on her face. At this time the total white blood cell count was 7,000 per c.mm. and the differential count normal. She was readmitted again to Stracathro Hospital on November 18 and January 11, 1948, because of recurrent attacks of increasingly severe dyspnoea with cyanosis and stridor, and she died on January 14, 1948.

Summary of Relevant Macroscopic Findings.—There was no emaciation, jaundice, or enlargement of the superficial lymph nodes. In the neck a small mass of apparently normal thyroid tissue, approximately 2 cm. in diameter, was found on the left side of the trachea and oesophagus. There was no evidence on the right side of any thyroid tissue or recurrence of the tumour and the neighbouring lymph nodes appeared normal. The tracheal bifurcation and main bronchi were enveloped in a mass of tumour tissue which

did not appear to be of bronchial origin. The tumour mass, which was white and homogeneous at its periphery, showed central necrosis and haemorrhage and, in general, had the appearance of a mass of confluent lymph nodes. No primary bronchial tumour could be demonstrated in either lung. The stomach contained two hard, discrete neoplastic ulcers with rolled edges and excavated centres. One, measuring 3 cm. in diameter, was situated on the anterior wall of the stomach, towards the cardia, while the other, measuring 5 cm. in diameter (Fig. 12), was situated on the greater curvature, midway between the cardia and the pylorus. The intervening gastric mucosa was normal, as was the rest of the stomach. The remainder of the alimentary tract was normal and there was no enlargement of the regional lymph nodes. No metastases were visible in the liver or in any of the other abdominal organs. Permission to examine the brain was withheld.

Histology.—The thyroid tumour was highly cellular and anaplastic, consisting predominantly of round cells, which possessed scanty cytoplasm and rather pale staining nuclei. Scanty cells of reticulum cell type were present, as well as lymphocytes and moderate numbers of mitoses (Figs. 13 and 14). Examination of the ulcers in the stomach showed that they also were composed of anaplastic tumour tissue, consisting predominantly of round cells similar to those seen in the thyroid tumour. Here again lymphocytes, cells of reticulum cell type, and mitoses were present in small numbers. The tumour in both cases was situated in the submucosa and was infiltrating the deeper layers of the mucosa as well as the muscularis. The appearances of the mass at the tracheal bifurcation were essentially the same as those of the gastric and thyroid tumours, and there was no evidence of reticulin formation in any of the tumours.

The cytological appearances of the gastric tumours indicate that they are of the lymphoblastic variety of reticulosarcoma. The histological similarity between them, the mediastinal mass and the thyroid tumour, shows that the latter two are also foci of lymphoblastic reticulosarcoma. It is impossible to decide which of the four foci is the primary tumour or whether the disease is of multifocal origin, but the absence of involvement of the regional lymph nodes in the neck suggests that the thyroid tumour may not be the primary focus.

Discussion

In the past, great reliance seems to have been placed on the presence of a fine reticulin network, lying in close apposition to the tumour cells, as well as on the cytological appearances of the latter, as diagnostic criteria of reticulosarcoma of the thyroid. However, Robb-Smith (1938) demonstrated that by no means all reticulosarcomata produce reticulin, and that, whereas it is abundant in the polymorphic and dictyocytic varieties, it is virtually non-existent in the syncytial and lymphoblastic tumours.

It has been demonstrated in Case 1 that the presence of a reticulin network in an anaplastic thyroid tumour by no means excludes the possibility of that tumour being a carcinoma. Also, it is well known that the degree of anaplasia and pleomorphism shown by some malignant tumours makes it impossible to decide, from the cytological appearances alone, whether these tumours are of epithelial or mesenchymal origin.

Thus the conclusion is reached that the cytological appearances of an anaplastic thyroid tumour and the presence or absence of reticulin production in that tumour are unreliable as diagnostic criteria of reticulosarcoma of the thyroid. Although these features in a thyroid tumour may raise the possibility of its being a reticulosarcoma, it is believed that the final diagnosis should not be made on examination of the thyroid tumour alone and that recourse should be had to collateral evidence

where possible. The absence of this collateral evidence has possibly led, in part at least, to the considerable variation in the incidence of these tumours between different observers. With the exception of Case 34032 of the Massachusetts General Hospital and Case 3 above, both of which came to necropsy, all the cases cited in the introduction to this paper were apparently diagnosed on histological examination of the thyroid tumour alone. Two of the cases presented by Rice (1932) and four of those presented by Kellett and Sutherland (1949) were dead at the time of these authors' reports, but no necropsies were performed.

It is only by the demonstration of other foci of tumour, which by their situation and macroscopical and microscopical appearances leave little doubt that they are foci of reticulosarcoma, that the final diagnosis can be made with any degree of certainty. This is well demonstrated in Cases 2 and 3, in which the diagnosis of reticulosarcoma, considered as a possibility at the time of the examination of the thyroid tumours, was confirmed only by the subsequent finding of collateral evidence of the type mentioned above.

Summary

Attention is drawn to the difficulty of making a histological distinction between reticulosarcomata and certain anaplastic carcinomata of the thyroid. The relevant literature is reviewed and the opinion expressed that the frequency with which a diagnosis of reticulosarcoma of the thyroid is made varies greatly between observers according to the diagnostic criteria employed. One case of anaplastic carcinoma and two cases of authenticated reticulosarcoma of the thyroid are presented. From a study of these cases, the conclusion is reached that the cytological appearances, as well as the presence or absence of reticulin, are unreliable diagnostic criteria and should not be used as a means of making the final diagnosis, the latter being withheld in the absence of collateral evidence, the nature of which is described.

I wish to express my thanks to Professor J. S. Young for placing much of the material at my disposal; to Mr. F. J. Sambrook Gowar for the clinical details of Case 1; to Mr. H. Wapshaw and Mr. N. J. Logie for the clinical details of Case 2; to Professor D. F. Cappell for allowing me access to the material of the thyroid tumour in Case 2; to the Medical Superintendent of Stracathro Hospital for the clinical details of Case 3; to Dr. J. A. F. McLean for details of the post-mortem examination in Case 3; to Mr. N. Mowat for technical assistance; and to Mr. W. H. Carnie for the photographs.

References

Joll, C. A. (1941). *Postgrad. med. J.*, **17**, 166.
Kellett, H. S., and Sutherland, T. W. (1949). *J. Path Bact.*, **61**, 233.
Massachusetts General Hospital. (1939) (Case 25421.) *New Engl. med. J.*, **221**, 617.
—— (1941). (Case 27451.) Ibid., **225**, 752. (Case 27471.) Ibid., **225**, 833.
—— (1948). (Case 34032.) Ibid., **238**, 97.
Portmann, U. V. (1940). *Surg. Gynec. Obstet.*, **70**, 185.
Rice, C. O. (1932). *Virchows Arch.*, **286**, 459.
Robb-Smith, A. H. T. (1938). *J. Path. Bact.*, **47**, 457.
Vaux, D. M. (1937). Ibid., **44**, 463.

J. clin. Path. (1952), **5**, 190.

SERUM COPPER AND ITS RELATIONSHIP TO SERUM IRON IN PATIENTS WITH NEOPLASTIC DISEASE

BY

ROBERT PIRRIE

From the Muirhead Department of Medicine, the University and Royal Infirmary, Glasgow

(RECEIVED FOR PUBLICATION JULY 10, 1951)

The importance of serum copper in the transport of copper throughout the body was established by Sachs, Levine, Schmit, and Hughes (1941) when they observed that the oral administration of varying amounts of copper sulphate was followed by a definite rise in the copper content of the serum. Later Yoshikawa, Hahn, and Bale (1942) obtained a similar result by feeding radio-active copper salts to normal and anaemic dogs. After its ingestion the tagged metal rapidly appeared in the serum, reaching its peak concentration within two to five hours. These observations indicate that the role of serum copper in copper metabolism is analogous to that of serum iron in iron metabolism, and, by permitting valid conclusions to be drawn from estimations of serum copper levels, have facilitated the study of copper metabolism in man.

The purpose of this paper is to present the values of serum copper obtained in normal subjects and in patients with neoplastic disease unaccompanied by haemorrhage, and to compare the iron and copper concentrations in the serum of these patients.

Methods

Serum copper was determined by a minor modification of the technique described by Cartwright, Jones, and Wintrobe (1945). This method consists of the triple extraction of the trichloracetic acid precipitate of serum and the development of the coloured copper-dithiocarbamate complex, the resultant colour being measured directly in the aqueous extract. Difficulty was encountered in obtaining optically clear aqueous extracts for photometry, however, and extraction of the coloured complex with amyl alcohol, as originally suggested by McFarlane (1932), was therefore employed. The procedure finally adopted was as follows. The aqueous phase, obtained by following the procedure of Cartwright *et al.*, was made up to a volume of 20 ml. with distilled water in a graduated test-tube, 10 ml. of amyl alcohol added, and the test tube shaken vigorously for two minutes. The amyl alcohol phase was then separated by centrifugalization and transferred by a Pasteur pipette to a thoroughly dried 1 cm. optical cell. Colour was measured in a Spekker absorptiometer using the Ilford spectrum filter, No. 601. Clear extracts of the coloured copper complex were consistently obtained when this procedure was followed.

Serum iron was estimated by the method of Fitzpatrick and Howells (1949), by whom it has already been described in detail. The Haldane method was used for haemoglobin determination.

Results

Copper was estimated in triplicate in five different sera (Table I) and the results showed good agreement, the maximum difference being 4.5%.

TABLE I

TRIPLICATE ESTIMATIONS OF SERUM COPPER

Serum	Copper Content (μg. %)		
1	89	93	93
2	151	150	153
3	326	326	324
4	115	112	112
5	110	115	115

In 20 healthy adult males, the mean and its standard error of serum copper values were 105 ± 5.74 μg. % with a range of 74 to 151 μg. %. The corresponding values in 20 healthy adult females were 120 ± 6.72 μg. % and 84 to 168 μg. %. This difference between the sexes was not significant. These results compare favourably with those already reported (Table II); only in the series of Nielsen (1944a) and Munch-Petersen (1948) is the observed sex difference in serum copper significant.

TABLE II

SUMMARY OF PUBLISHED DATA ON SERUM COPPER IN NORMAL SUBJECTS

Investigator	Serum Copper (μg. %)					
	Males			Females		
	No.	Mean	Range	No.	Mean	Range
Nielsen (1944a)	30	110	75–165	100	123	75–165
Cartwright *et al.* (1945)	25	116	92–134	25	131	103–159
Munch-Petersen (1948)	50	108	81–164	50	118	73–152
Cartwright *et al.* (1948)	52	114	86–141	53	123	87–161
Brendstrup (1948)	40	113	84–162	40	120	89–152
Present investigation	20	105	74–151	20	120	84–168

Serum copper and serum iron were determined in 19 patients with malignant disease unaccompanied by haemorrhage, and the results are set out in Table III. The mean value for serum copper in these patients was 223 ± 14.18 μg. %; the difference between serum copper levels in these patients and those of normal subjects was highly significant. The values for serum iron had a mean of 67 ± 5.96 μg. % and were significantly lower than those obtained in normal subjects by the potassium ferricyanide method (Pirrie, 1952). In Fig. 1 serum copper results are plotted against serum iron results, the latter being grouped in 20 μg. units. The relationship between the two is approximately linear, and the individual results for serum copper were found to show a statistically significant inverse correlation to those for serum iron. No significant correlation between serum copper and haemoglobin was established in this series. Thus, of the five patients whose haemoglobin and red cell counts were within normal limits, three had elevated serum copper and lowered serum iron.

TABLE III

SERUM COPPER AND SERUM IRON IN PATIENTS WITH NEOPLASTIC DISEASE UNACCOMPANIED BY HAEMORRHAGE

Diagnosis	Age (yrs.)	Sex	Hb. (%)	R.B.C. ($\times 10^6$/ c.mm.)	Serum Copper (μg. %)	Serum Iron (μg. %)
Carcinoma of lung	54	M.	114	5·78	184	73
" "	61	M.	64	—	325	40
" "	62	M.	74	4·25	211	96
" "	38	M.	74	3·92	326	65
" "	39	M.	89	—	178	57
" "	43	M.	70	3·85	211	67
" "	67	M.	89	5·01	311	43
" "	40	M.	58	3·65	228	35
" "	63	M.	122	6·51	151	110
Sarcoma of femur	35	M.	70	3·94	262	56
Carcinoma of prostate	69	M.	102	5·49	174	95
" "	71	M.	106	6·12	193	81
" "	58	M.	76	—	138	105
Carcinoma of tongue	67	M.	88	5·02	178	43
Carcinoma of penis	71	M.	105	5·62	124	105
Carcinoma of oesophagus	59	M.	84	4·91	263	30
Carcinoma of breast	67	F.	104	5·84	213	74
" "	58	F.	82	4·45	293	40
" "	38	F.	106	5·37	245	50

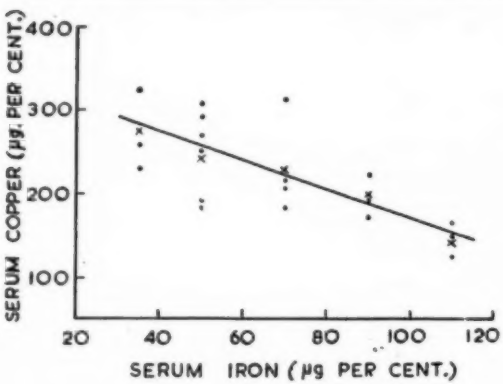


FIG. 1.—The relationship between serum copper and serum iron in patients with malignant disease unaccompanied by haemorrhage. Individual results of serum copper (●). Mean serum copper of serum iron groups (x).

Discussion

The effect of various diseases upon serum copper has already been studied. Hypocupraemia is rarely found and is not apparently peculiar to any particular clinical entity. Hypercupraemia occurs in thyrotoxicosis (Nielsen, 1944b), in infectious mononucleosis (Munch-Petersen, 1948), in both acute and chronic infections (Cartwright, Lauritsen, Jones, Merrill, and Wintrobe, 1946), and in pregnancy, in which it appears after the third month and persists until four to seven weeks after parturition (Nielsen, 1944c). In addition, Cartwright, Huguley, Ashenbrucker, Fay, and Wintrobe (1948) have described its occurrence in iron deficiency anaemia, in nephritis with anaemia, and in leukaemia and lymph node disorders.

The significance of hypercupraemia and of the inverse correlation between the degrees of hypercupraemia and hypoferraemia, which were observed in these patients with neoplastic disease, is difficult to assess at present. The assumption that the two metals compete for the same metal-combining protein in the plasma is unjustified in the light of the work of Holmberg and Laurell (1947). The iron-combining protein is a β_1-globulin, and, although it can combine with copper *in vitro*, (Surgenor, Koechlin, and Strong, 1949) there is no evidence to show that it does so *in vivo*. On the contrary, Holmberg and Laurell (1947) have shown that the main part of native serum copper is found in the α-globulin fraction of the serum. In this connexion, it is interesting to note that there is a conspicuous increase in α_2-globulins in the serum of patients with neoplastic disease (Shedlovsky and Scudder, 1942; Seibert, Seibert, Atno, and Campbell, 1947).

The occurrence of anaemia in these patients even in the absence of blood loss demonstrates that haemoglobin synthesis is depressed or deranged in the presence of neoplastic disease. A reduced demand for copper, an essential catalyst in haemoglobin synthesis, may be the explanation of the observed hypercupraemia.

Summary

Mean values of 105 ± 5.74 μg. % and 120 ± 6.72 μg. % were established for serum copper in 20 healthy adult males and 20 healthy adult females respectively. This sex difference was not significant.

Serum copper levels in 19 patients with neoplastic disease unaccompanied by haemorrhage were significantly higher than normal, and serum iron was significantly lower.

A significant inverse correlation between serum copper and serum iron was observed in these patients.

These findings are briefly discussed.

While this investigation was being carried out, the author was Faulds Research Fellow in Medicine of Glasgow University and part of the expenses incurred was provided by the Rankin Fund.

I wish to thank Professor L. J. Davis for his advice during the preparation of this paper.

References

Brendstrup, P. (1948). *Ugeskr. Laeg.*, **110**, 945.
Cartwright, G. E., Huguley, C. M., Ashenbrucker, H., Fay, J., and Wintrobe, M. M. (1948). *Blood*, **3**, 501.
—— Jones, P. J., and Wintrobe, M. M. (1945). *J. biol. Chem.*, **160**, 593.
—— Lauritsen, M. A., Jones, P. J., Merrill, I. M., and Wintrobe, M. M. (1946). *J. clin. Invest.*, **25**, 65.
Fitzpatrick, J., and Howells, K. W. (1949). *Journal of Clinical Pathology*, **2**, 290.
Holmberg, C. G., and Laurell, C. B. (1947). *Acta chem. scand.*, **1**, 944.
McFarlane, W. D. (1932). *Biochem. J.*, **26**, 1022.
Munch-Petersen, S. (1948). *Acta med. scand.*, **131**, 588.
Nielsen, A. L. (1944a). Ibid., **118**, 87.
—— (1944b). Ibid., **118**, 431.
—— (1944c). Ibid., **118**, 92.
Pirrie, R. (1952). *Journal of Clinical Pathology*, **5**, 10.
Sachs, A., Levine, V. E., Schmit, A., and Hughes, R. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 192.
Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W. (1947). *J. clin. Invest.*, **26**, 90.
Shedlovsky, T., and Scudder, J. (1942). *J. exp. Med.*, **75**, 119.
Surgenor, D. M., Koechlin, B. A., and Strong, L. E. (1949). *J. clin. Invest.*, **28**, 73.
Yoshikawa, H., Hahn, P. F., and Bale, W. F. (1942). *J. exp. Med.*, **75**, 489.

J. clin. Path. (1952), **5**, 194.

BLOOD SERUM PROTEIN-BOUND CARBOHYDRATE AFTER INJURY

BY

J. W. KEYSER

From the Department of Pathology and Bacteriology, Welsh National School of Medicine, Cardiff

(RECEIVED FOR PUBLICATION JULY 13, 1951)

In earlier publications (Keyser, 1949, 1950) we reported increases in the level of a substance giving the tryptophane-perchloric acid reaction (Cohen, 1944; Seibert, Pfaff, and Seibert, 1948) in the blood serum of six burn cases. No attempt was made at the time to identify the reacting substance, which is generally assumed to be a polysaccharide. The present investigation was undertaken to find out whether a rise in the level of serum protein-bound carbohydrate (protein sugar, non-glucosamine polysaccharide) after injury could be demonstrated by a more specific method and, if so, how much of it is contributed by the mucoprotein fraction studied by Winzler, Devor, Mehl, and Smyth (1948). It is possible that this mucoprotein fraction consists largely of the polypeptide-like substances occurring in trichloroacetic acid filtrates of serum (Hahn, 1921; Wolff, 1921) and precipitable therefrom by tungstic or phosphotungstic acid. The serum level of these polypeptides was reported to be raised after surgical operations and after burning (Cristol and Puech, 1929; Fiessinger, 1934; Goiffon and Spaey, 1934a, 1934b; Duval, Roux, and Goiffon, 1934). For convenience the carbohydrate measured will be referred to as "protein sugar."

Methods

Blood was obtained by venepuncture, the serum being separated as soon as possible and kept frozen until the analyses were performed. In agreement with the findings of Seibert and Atno (1946), control experiments on two normal people showed no significant difference between the fasting and non-fasting levels of serum protein sugar, nor was the mucoprotein sugar significantly altered by feeding. Consequently no effort was made to obtain blood only in the fasting or non-fasting state. For the estimation of total serum protein sugar the proteins were separated by alcoholic precipitation as follows.

Serum, 1 ml., was added drop by drop to 19 ml. of absolute ethyl alcohol and the mixture shaken vigorously. The precipitated protein was spun down, the alcohol decanted, and the tube inverted and allowed to drain for a few minutes. The protein was then washed by boiling with two successive 10 ml. portions of 95% (by volume) ethyl alcohol, the precipitate being broken up and stirred with a fine glass rod during the process. After each washing the protein was spun down, the alcohol decanted, and the tube allowed to drain as before. Finally the protein was dissolved in 0.125N sodium hydroxide to a volume of 10 ml. One portion (1 or 2 ml.) of the alkaline protein solution was taken for Kjeldahl digestion. Duplicate 0.5 ml. portions were taken for analysis by the orcinol method (Tillmans and Philippi, 1929; Sørensen and Haugaard, 1933),

essentially as described by Rimington (1940). Care was taken to avoid exposure to light during and after the heating. No attempt was made to carry out a protein "blank" by heating with sulphuric acid in the absence of orcinol, since it has been shown (Sørensen, 1936; Hewitt, 1937) that the slightly coloured solutions obtained in this way do not represent true blanks and are a source of error. Reagent blanks were performed. A standard calibration curve, prepared with a solution of equal parts by weight of galactose and mannose, was checked at frequent intervals.

Mucoprotein was estimated by the method of Winzler, Devor, Mehl, and Smyth (1948). To 2 ml. of serum 8 ml. of 0.75M perchloric acid was added and the mixture vigorously shaken. After 10 minutes the precipitated proteins were filtered off with a Whatman No. 50 filter paper (9 cm.). The mucoprotein was precipitated from duplicate 2 ml. portions of the filtrate by the addition of 0.4 ml. of a 5% solution of phosphotungstic acid in 2N hydrochloric acid, and spun down after 15 minutes. The precipitate was washed first with 1 ml. of the phosphotungstic acid solution and then with 2 ml. of 95% (by volume) ethyl alcohol, being thoroughly broken up and stirred with a fine glass rod in each washing. The mucoprotein was finally dissolved in 0.5 ml. of 0.125N sodium hydroxide for analysis by the orcinol method.

Results

The findings are summarized in Table I. Normal values for total serum protein sugar and mucoprotein sugar found in this laboratory are given in Table II, and are mostly in the range of published figures. It is evident that after injury there was a considerable rise in the concentration of both total protein sugar and mucoprotein sugar. The rise in the former is in accord with the observations of Shetlar, Bryan, Foster, Shetlar, and Everett (1949), which came to my notice while the present work was in progress. They found that experimental injury to animals (e.g., the production of sterile abscesses, laparotomy, and so on) resulted in a more or less pronounced rise in the serum polysaccharide level, as measured by a tryptophane method (Sheppard and Everett, 1937; Shetlar, Foster, and Everett, 1948). They do not appear, however, to have estimated the mucoprotein fraction.

The mucoprotein figures shown in Tables I and II are almost certainly lower than the true values, as it has been shown by Winzler and his colleagues that considerable amounts of mucoprotein are adsorbed on the precipitate obtained when perchloric acid is added to serum. These losses are smallest when the serum proteins are lowered. In our cases the serum proteins (Table I) were not sufficiently lowered to account for the high mucoprotein values found. It is interesting to note that the proportion of the total increase in protein sugar contributed by the mucoprotein fraction varied considerably and clearly tended to be highest when the total protein sugar was only slightly raised. Indeed, the mucoprotein sugar was sometimes raised when the total protein sugar was within normal limits. This has been found also in an investigation (to be published) of patients treated with nitrogen mustard or by radiation therapy.

In all but one of our cases the tryptophane-perchloric acid reaction was also carried out: it parallelled roughly the total protein sugar.

Discussion

These results confirm and extend those previously obtained by us with the tryptophane-perchloric acid method, which were doubtless due to a rise in the serum protein sugar.

TABLE I

TOTAL PROTEIN AND MUCOPROTEIN SUGAR IN BURNS AND FRACTURES

Case No.	Age	Sex	Type of Injury	Days after Injury	Total Serum Protein (g./100 ml.)	Total Serum Protein Sugar as Galactose-mannose (mg./100 ml.)	Serum Mucoprotein Sugar as Galactose-mannose (mg./100 ml.)
1	23	M.	Burns and fractures	2	6·1	115	—
				4	5·9	160	41
				10	5·8	232	64
				26	6·4	220	54
				81	7·0	142	21
				199	—	126	18
2	10/12	M.	Burns	3	6·3	143	22
3	47	M.	Burns	1	6·1	156	—
				7	5·9	186	45
4	27	M.	Burns	1	6·5	148	—
				7	7·1	186	31
5	76	F.	Fracture	4	7·0	170	26
6	54	M.	Fracture	12	6·3	155	22
				22	7·1	141	19
7	17½	F.	Fracture	1	6·7	132	16
				5	7·4	221	26
8	79	F.	Fracture	5	5·5	134	21
				9	6·3	164	23
9	52	M.	Fracture	10	6·8	160	25
				17	6·0	140	18
10	70	F.	Fracture	59	6·6	146	18

TABLE II

TOTAL SERUM PROTEIN AND MUCOPROTEIN SUGAR IN NORMALS

	Total Serum Protein Sugar* (18 people)	Mucoprotein Sugar* (13 people)
Range	102–139	10·0–13·0
Mean	119	11·9
Standard deviation	12	1·2

* As mg. galactose-mannose per 100 ml. serum.

It seems likely that the increases in serum α-globulin reported by various workers after experimental burning in animals (Perlmann, Glenn, and Kaufman, 1943; Chanutin and Gjessing, 1946) and in human burns and fracture cases (Shedlovsky and Scudder, 1942) are due, in part at least, to the mucoprotein fraction (cf. electrophoretic data of Mehl, Humphrey, and Winzler, 1949; Mehl, Golden, and Winzler, 1949; and Petermann and Hogness, 1948).

Seibert and her colleagues (Seibert, Pfaff, and Seibert, 1948; Seibert, Seibert, Atno, and Campbell, 1947) have suggested that elevation of serum polysaccharide is associated with tissue destruction. An alternative hypothesis is that it is associated with healing processes or proliferation (Shetlar, Bryan, Foster, Shetlar, and Everett, 1949; Shetlar, Erwin, and Everett, 1950; Shetlar, Foster, Kelly, Shetlar, Bryan, and Everett, 1949). Although our findings are consistent with the "tissue destruction" hypothesis, the prolonged elevation of total protein sugar and mucoprotein sugar in Case 1 would suggest some connexion with healing. (It must be emphasized that the destruction we have in mind is not merely that due to local trauma, but is rather the general metabolic response to injury implied in the well-known conception of a katabolic phase of negative nitrogen balance, and implied also in the suggestion of Croft and Peters (1945) that after burning there is a "raiding" of the tissue proteins for one or two amino acids particularly needed for the formation of new skin protein.) However, this prolonged elevation might have been due partly to skin-grafting necessitated by the presence of unhealed areas, though the burn itself was completely healed by the forty-second day after the injury. Unfortunately it was not practicable to study the other cases for more than a few days. As far as could be ascertained Cases 5 to 10 were completely afebrile and the majority of all our cases were uncomplicated by the presence of disease.

Apart from the hypotheses mentioned above, it might be that after injury there is a retention of carbohydrate-rich protein fractions or a failure to metabolize them. This possibility cannot at present be excluded.

Summary

A study of 10 cases of burns and fractures revealed a well-marked rise in the blood serum protein-bound and mucoprotein-bound carbohydrate (protein sugar, non-glucosamine polysaccharide) after injury. In one patient who was followed up for several months the total protein sugar and mucoprotein sugar reached a maximum several days after injury; but whereas the total protein sugar had fallen to normal levels after 11 weeks, the mucoprotein sugar was still high after 28 weeks.

I wish to thank the many people who helped me to obtain blood specimens, and the surgeons who allowed me to study their cases. I am grateful also to Professor J. Gough for his help and encouragement.

References

Chanutin, A., and Gjessing, E. C. (1946). *J. biol. Chem.*, **165**, 421.
Cohen, S. S. (1944). Ibid., **156**, 691.
Cristol, P., and Puech, A. (1929). Cited by Goiffon and Spaey, 1934.
Croft, P. B., and Peters, R. A. (1945). *Lancet*, **1**, 266.
Duval, P., Roux, J.- C., and Goiffon, R. (1934). *Pr. méd.*, **42**, 1785.
Fiessinger, N. (1934). Ibid., **42**, 1787.
Goiffon, R., and Spaey, J. (1934a). *Bull. Soc. Chim. biol.*, **16**, 1675.
—— —— (1934b). *C.R. Soc. Biol., Paris*, **115**, 711.
Hahn, A. (1921). *Biochem. Z.*, **121**, 262.
Hewitt, L. F. (1937). *Biochem. J.*, **31**, 360.
Keyser, J. W. (1949). *Nature, Lond.*, **164**, 889.
—— (1950). *Journal of Clinical Pathology*, **3**, 106.
Mehl, J. W., Golden, F., and Winzler, R. J. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 110.
—— Humphrey, J., and Winzler, R. J. (1949). Ibid., **72**, 106.
Perlmann, G. E., Glenn, W. W. L., and Kaufman, D. (1943). *J. clin. Invest.*, **22**, 627.

Petermann, M. L., and Hogness, K. R. (1948). *Cancer*, **1**, 100, 104.
Rimington, C. (1940). *Biochem. J.*, **34**, 931.
Seibert, F. B., and Atno, J. (1946). *J. biol. Chem.*, **163**, 511.
—— Pfaff, M. L., and Seibert, M. V. (1948). *Arch. Biochem.*, **18**, 279.
—— Seibert, M. V., Atno, A. J., and Campbell, H. W. (1947). *J. clin. Invest.*, **26**, 90.
Shedlovsky, T., and Scudder, J. (1942). *J. exp. Med.*, **75**, 119.
Sheppard, F., and Everett, M. R. (1937). *J. biol. Chem.*, **119**, lxxxix.
Shetlar, M. R., Bryan, R. S., Foster, J. V., Shetlar, C. L., and Everett, M. R. (1949). *Proc. Soc. exp. Biol., N.Y.*, **72**, 294.
—— Erwin, C. P., and Everett, M. R. (1950). *Cancer Res.*, **10**, 445.
—— Foster, J. V., and Everett, M. R. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 125.
—— —— Kelly, K. H., Shetlar, C. L., Bryan, R. S., and Everett, M. R. (1949). *Cancer Res.*, **9**, 515.
Sørensen, M. (1936). *Biochem. Z.*, **287**, 140.
—— and Haugaard, G. (1933). Ibid., **260**, 247.
Tillmans, J., and Philippi, K. (1929). Ibid., **215**, 36.
Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M. (1948). *J. clin. Invest.*, **27**, 609.
Wolff, E. (1921). *Ann. Méd.*, **10**, 185.

J. clin. Path. (1952), **5**, 199.

INTRATHECAL STREPTOMYCIN AND CEREBROSPINAL GLUCOSE ESTIMATION

BY

M. J. H. SMITH

From the Department of Chemical Pathology, King's College Hospital Medical School, London

(RECEIVED FOR PUBLICATION JANUARY 1, 1952)

The determination of cerebrospinal fluid glucose is important in the diagnosis and prognosis of tuberculous meningitis. The restoration to normal of the characteristically low glucose values is considered to reflect the success of streptomycin therapy. Intrathecal injection of the antibiotic is usually employed in tuberculous meningitis, and attention has been drawn to the fact that streptomycin itself has reducing properties (Cruickshank, 1951) and may interfere with the accurate measurement of cerebrospinal fluid glucose. Alvaro and Nicola (1948), using the Hagedorn-Jensen method, reported that various streptomycin preparations were strongly reducing, and that 1 mg. of anhydrous streptomycin base was equivalent to values up to 4.5 mg. of glucose. It was stated by Wilson (1951) that the intrathecal injection of antibiotics may give temporary false sugar values, so that estimations carried out during and for an indeterminate time after this form of treatment are invalidated.

These considerations suggested an investigation of the reducing powers of streptomycin and dihydrostreptomycin with special reference to the method of measuring cerebrospinal fluid glucose (Nelson, 1944) used in this laboratory. In addition, cerebrospinal fluid glucose results from a number of cases of tuberculous meningitis treated with intrathecal streptomycin were examined for any evidence of falsely high values.

Experimental

Streptomycin calcium chloride (Glaxo) and dihydrostreptomycin sulphate (Glaxo) were used in the present work. Both these substances are hygroscopic and a determination of moisture was made on each sample used for analysis. About 500 mg. of the sample were accurately weighed in a porcelain boat and the loss in weight determined after heating for three hours at 60° C. over phosphorus pentoxide under a reduced pressure of 10 mm. Hg.

Solutions of streptomycin calcium chloride and dihydrostreptomycin sulphate in distilled water, ranging in concentration from 10 to 500 mg. per 100 ml. of the respective bases, were prepared and 1 ml. of copper reagent (Nelson, 1944) was added to 1 ml. of a 1 in 10 dilution of each concentration, and to 1 ml. of a 4 mg. per 100 ml. solution of glucose. The mixture was immersed in boiling water for 20 minutes, cooled, and 2 ml. of the Nelson arseno-molybdate chromogen added, followed by 8.5 ml. water. The optical

densities were measured in a Spekker photoelectric absorptiometer against a reagent blank in which 1 ml. of water was substituted for the streptomycin or glucose solution, using a 1 cm. cell and an Ilford spectrum red No. 608 filter.

Results

The reducing power of anhydrous streptomycin base in terms of milligrammes of glucose, using the Nelson method of estimation, is shown in Fig. 1; 1 mg. of glucose is equivalent to approximately 8 mg. of anhydrous streptomycin base; dihydrostreptomycin showed no reducing action in the Nelson method.

Typical cerebrospinal fluid glucose values observed in a case of treated tuberculous meningitis (a 3½-year-old child) are shown in Fig. 2. The treatment, which began on Day 1, consisted of daily intrathecal injections of 50 mg. of streptomycin

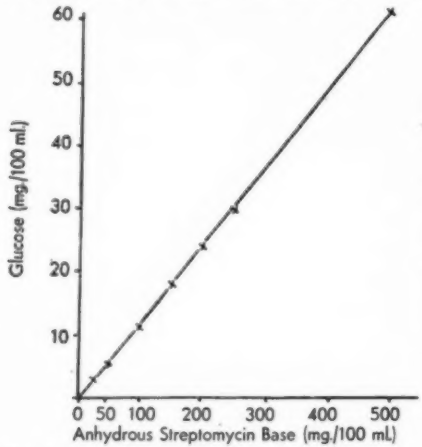


FIG. 1.—Glucose equivalent of anhydrous streptomycin base by Nelson's method.

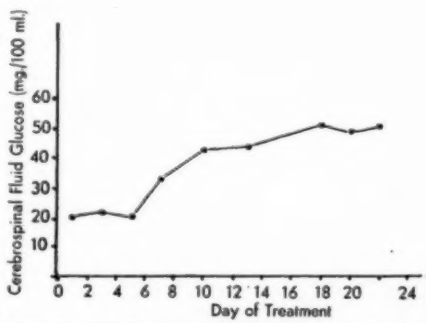


FIG. 2.—Cerebrospinal fluid glucose results in a 3½-year-old child treated with 50 mg. of daily intrathecal streptomycin calcium chloride from Day 1 onwards.

calcium chloride and 6 units of streptokinase, twice-daily intramuscular injections of 0.25 g. of streptomycin calcium chloride, and 1.5 g. of para-aminosalicylic acid every two and a half hours by mouth. Samples of cerebrospinal fluid for glucose analysis were withdrawn immediately before the injection of streptomycin and fluoride was added to inhibit glycolysis.

Discussion

The ratio 8 mg. of anhydrous streptomycin base to 1 mg. of glucose in the Nelson method means that a concentration of over 50 mg. per 100 ml. of streptomycin in cerebrospinal fluid is necessary before any significant interference with glucose measurements is produced. Samples of cerebrospinal fluid for analysis are normally taken 24 hours after the injection of 50 or 100 mg. of a streptomycin preparation. In both children and adults it was found (M.R.C. Report, 1948) that, one to three hours after the intrathecal injection of 100 mg. of streptomycin, the cerebrospinal fluid streptomycin level was between 75 and 200 mg. per 100 ml., but fell rapidly

between four and 10 hours and was between 0.2 and 1.6 mg. per 100 ml. (range 0.1 to 7.5) 24 hours after the injection. Russell and MacArthur (1950) could not detect any streptomycin in the cerebrospinal fluid 24 hours after the intrathecal injection of 50 mg. of streptomycin in a child.

It must be concluded that the intrathecal injection of streptomycin preparations in the doses normally employed will not give artificially high values for glucose in samples of cerebrospinal fluid obtained 24 hours after injection and analysed by the method of Nelson.

Confirmation of this conclusion was found when the cerebrospinal fluid glucose values of treated cases were examined; for example, in Fig. 2, which represents a typical case, there was a period of five days before the glucose figure began to rise significantly, whereas, if streptomycin affected the result, an elevation would have been observed much sooner. Wilson (1951) reported a case of *Staphylococcus aureus* meningitis in which the cerebrospinal fluid glucose rose from 33 mg. per 100 ml. to 55 mg. per 100 ml. 24 hours after the intrathecal injection of 100 mg. of dihydrostreptomycin sulphate, and suggested that the increased glucose value was due to the antibiotic. The method of estimation of cerebrospinal fluid glucose used by this author was not recorded, but in the present work dihydrostreptomycin was found to have no reducing action in the Nelson procedure. Alvaro and Nicola (1948), using the Hagedorn-Jensen method, stated that 1 mg. of streptomycin was equivalent to values of 0.32 to 4.5 mg. of glucose and quoted a value of 0.44 found by Nitti and Curci (1947). They also observed an immediate and steady rise in cerebrospinal fluid glucose values in cases treated with intrathecal streptomycin.

Summary

The reducing power of streptomycin and dihydrostreptomycin on the reagents used in the Nelson method for cerebrospinal fluid glucose has been investigated.

Dihydrostreptomycin has no reducing action, and approximately 8 mg. of anhydrous streptomycin base are equivalent to 1 mg. of glucose.

It is concluded that neither dihydrostreptomycin nor streptomycin will cause falsely high values for cerebrospinal fluid glucose in cases of tuberculous meningitis receiving these antibiotics by intrathecal injection.

I wish to express my gratitude to Dr. W. P. H. Sheldon for permission to publish the data in Fig. 2.

REFERENCES

Alvaro, M., and Nicola, M. (1948). *Riv. Clin. pediat.*, **46**, 372.
Cruickshank, D. B. (1951). *Recent Advances in Clinical Pathology*, ed. S. C. Dyke, 2nd ed., p. 43. London.
Medical Research Council (1948). Streptomycin in Tuberculosis Trials Committee. *Lancet*, **1**, 582.
Nelson, N. (1944). *J. biol. Chem.*, **153**, 375.
Nitti, V., and Curci, G. (1947). *Arch. Tisiol.*, **2**, 667.
Russell, S. J. M., and MacArthur, P. (1950). *Lancet*, **1**, 59.
Wilson, H. (1951). *Brit. med. J.*, **1**, 1206.

[Observations have also been made using the Hagedorn-Jensen method. Varying concentrations of streptomycin and dihydrostreptomycin were dissolved in pooled normal cerebrospinal fluid and titrated in the ordinary way. Using

dihydrostreptomycin (Glaxo) up to 1,000 μg. per ml., no change in reducing power of cerebrospinal fluid was found.

With streptomycin (Glaxo), two different batches gave excess reduction due to the streptomycin, equivalent to 28 and 36 mg. glucose per 100 ml. when present in a concentration of 1,000 μg. per ml.; with streptomycin concentrations of 100 and 200 μg. per ml., the rise was equivalent only to 4–6 mg. glucose per 100 ml.

Very high levels of streptomycin are only present a very short time after an intrathecal injection, and these findings, unlike those of Alvaro and Nicola, suggest that the administration of streptomycin is unlikely to cause any unreliability in cerebrospinal fluid sugar levels unless the cerebrospinal fluid is taken only a few hours after the last injection.—ED.]

J. clin. Path. (1952), **5**, 203.

THE CHOLESTEROL TOLERANCE TEST IN NORMAL INDIVIDUALS

BY

NAZEK I. FAHMY AND PAUL GHALIOUNGUI

From the Department of Clinical Medicine and the Abbassiah Faculty of Medicine, Cairo

(RECEIVED FOR PUBLICATION OCTOBER 19, 1951)

There are different opinions about the effect of a cholesterol-rich meal on the blood cholesterol.

Gardner and Gainsborough (1928), in their work on the cholesterol content of normal human plasma, came to the following conclusions on the so-called alimentary hypercholesterolaemia. (1) The level of the cholesterol content of human plasma in the fasting state can be raised or lowered by prolonged feeding with diets of high or low sterol content. Such changes are most marked as regards the cholesterol esters, while the free cholesterol remains practically constant. (2) Alimentary hypercholesterolaemia does not occur as the result of a single meal, a fact in accordance with Mjassnikow's (1926) views. Moreover, there is no connexion between the amount of sterol ingested and the cholesterol level of the plasma during digestion. They also concluded that the plasma cholesterol under normal and pathological conditions must be estimated in the fasting state. This is also the opinion of McEachen and Gilmour (1932), of McClure and Huntsinger (1928), of Gardner (1932), and of Bruger and Somach (1932), who observed physiological variations in the hourly blood cholesterol levels that led them to the conclusion that blood samples must be taken before breakfast.

Nepveux and Uhry mention (1936) that Goodman, Grigaut, Bacmeister, Laudat, McClure, and Huntsinger observed an elevation of the blood cholesterol parallel to the curve of lipaemia after the ingestion of any type of food, but especially after the absorption of cholesterol and egg yolk. They also mention (1936) that Leites, Martini, Bruger, and Oeter differentiated between digestive hypercholesterolaemia and alimentary hypercholesterolaemia. The former occurs without any dietary intervention, since the mere exciting of digestive secretions by the injection of dilute hydrochloric acid in the duodenum can provoke it. It is observed from the beginning of the evacuation of the stomach, whereas hypercholesterolaemia due to intestinal absorption comes on later.

Since cholesterol appears more and more related to vascular changes, it would be of real value to estimate the individual's cholesterol tolerance early in life. Should this tolerance be poor, one could attempt by a cholesterol-poor diet to maintain a normal blood cholesterol level (Burns, 1948).

Our attention was attracted to the effect of meals on the blood cholesterol by 15 cases (Table I) in which the blood taken before lunch but five hours after break-

fast showed unusually high values (ranging from 243.9 mg. to 200 mg., average 219.2 mg. %), as compared with the normal fasting blood cholesterol in Egyptians which, in our experience, is 115 mg. to 199 mg. %.

TABLE I

EFFECT OF MEALS ON BLOOD CHOLESTEROL

No.	Sex	Total (mg./100 ml.)
1	Male	212·7
2	,,	217·3
3	,,	204·4
4	,,	230·0
5	,,	212·7
6	,,	227·3
7	,,	208·3
8	,,	239·2
9	,,	200·0
10	,,	204·1
11	Female	210·7
12	,,	243·9
13	,,	220·7
14	,,	217·4
15	,,	240·3

A decision was first made to estimate the cholesterol value before breakfast and before the midday meal, i.e., five hours later, in the same individuals. As can be seen from Table II, it was higher in the later specimen, and it was therefore decided to estimate the effect of a standard breakfast.

TABLE II

RESULTS FASTING AND FIVE HOURS AFTER BREAKFAST IN THE SAME INDIVIDUALS

No.	Sex	Condition	Total (mg./100 ml.)
1	Male	Fasting	181·8
		5 hrs. after breakfast	257·7
2	,,	Fasting	173·0
		5 hrs. after breakfast	212·7
3	,,	Fasting	192·3
		5 hrs. after breakfast	212·7

Experimental Procedure

Blood was taken in the morning before the ingestion of any food; a meal was then given consisting of three eggs, 50 g. butter, 50 g. cheese, 200 g. milk, 190 g. bread, and 80 g. cooked beans. The average amount of cholesterol in one Egyptian egg yolk was found by chemical analysis to be 351.6 mg., and the average weight of one yolk is 16 g. Three hours and five hours after this meal blood was again taken. Exactly five hours after breakfast five subjects were given a lunch consisting of 200 g. rice, 100 g. meat, 200 g. potatoes, two oranges, and 188 g. bread. Three hours after taking this meal (eight hours after breakfast) blood specimens were again withdrawn. In one case only another specimen was taken four and a half hours after lunch.

TABLE III

RESULTS FASTING AND AT SPECIFIED INTERVALS AFTER MEALS

No.	Age	Sex	Fasting	Hours after Breakfast		Hours after Lunch	
				3 Hours	5 Hours	3 Hours	4½ Hours
1	24	F.	161·3	208·3	215·5	169·4	
2	30	M.	192·3	200·0	212·7	167·2	
3	30	M.	181·8	166·6	189·4	175·0	
4	32	M.	181·8	217·4	257·7	227·3	
5	39	M.	173·0	181·8	212·7	188·6	238·1

It was then decided to follow the effect of the standard breakfast alone for seven hours without the complicating factor of the lunch.

Tables IV, V, VI, and VII illustrate these results in 10 normal subjects for total, free, and esterified cholesterol respectively.

TABLE IV

EFFECT OF STANDARD BREAKFAST IN TEN NORMAL SUBJECTS FOR TOTAL CHOLESTEROL

No.	Sex	Age	Fasting	Hours after Breakfast		
				3	5	7
1	M.	28	195·3	208·3	212·7	190·1
2	M.	75	190·8	173·0	219·2	186·5
3	M.	22	161·3	157·2	175·5	172·4
4	M.	32	185·1	172·4	192·3	181·8
5	M.	20	147·1	167·7	190·8	147·0
6	M.	33	147·7	142·8	188·6	163·9
7	M.	38	175·5	196·1	208·3	156·2
8	M.	30	133·3	159·0	169·4	121·9
9	M.	30	133·3	144·9	161·3	147·1
10	M.	14	133·3	133·3	140·8	125·0
Average			160·3	165·5	185·9	159·2

TABLE V

EFFECT OF STANDARD BREAKFAST ON 10 NORMAL SUBJECTS FOR FREE CHOLESTEROL

No.	Sex	Age	Fasting	Hours after Breakfast		
				3	5	7
1	M.	28	130·8	139·4	150·2	143·6
2	M.	75	132·0	121·2	175·4	151·8
3	M.	22	135·4	123·9	143·8	126·9
4	M.	32	131·1	121·2	127·0	114·3
5	M.	20	116·7	129·3	155·1	94·5
6	M.	33	116·0	102·8	156·3	117·0
7	M.	38	125·5	151·7	169·8	108·6
8	M.	30	102·1	125·2	150·2	101·1
9	M.	30	100·0	122·6	139·6	126·9
10	M.	14	100·0	113·9	108·5	111·2
Average			118·6	125·1	147·9	119·6

TABLE VI

EFFECT OF STANDARD BREAKFAST ON 10 NORMAL SUBJECTS FOR ESTERIFIED CHOLESTEROL

No.	Sex	Age	Fasting	Hours after Breakfast		
				3	5	7
1	M.	28	64·5	68·9	62·5	46·5
2	M.	75	58·8	51·8	43·8	34·7
3	M.	22	25·9	33·3	31·7	45·5
4	M.	32	54·0	51·2	65·3	67·5
5	M.	20	30·4	38·4	35·7	42·5
6	M.	33	31·7	40·0	32·3	46·9
7	M.	38	50·0	44·4	38·5	47·6
8	M.	30	31·2	33·8	19·2	20·8
9	M.	30	33·3	22·3	21·7	20·2
10	M.	14	33·3	19·4	32·3	13·8
Average			41·3	40·4	38·3	38·6

Table VII illustrates the cholesterol ester as percentages of the total in the fasting state three, five, and seven hours after the ingestion of the standard breakfast in the same 10 normal individuals.

TABLE VII

CHOLESTEROL ESTER AS PERCENTAGES OF TOTAL CHOLESTEROL IN 10 NORMAL SUBJECTS AT SPECIFIED INTERVALS AFTER STANDARD BREAKFAST

No.	Sex	Age	Fasting	Hours after Breakfast		
				3	5	7
1	M.	28	33·0	33·1	29·4	24·5
2	M.	75	30·8	29·9	19·8	18·6
3	M.	22	16·1	11·2	18·6	26·3
4	M.	32	29·1	29·7	33·9	37·1
5	M.	20	20·6	22·9	17·8	28·9
6	M.	33	21·4	28·0	17·1	28·6
7	M.	38	28·4	18·5	22·6	30·5
8	M.	30	30·5	21·2	11·3	17·1
9	M.	30	24·9	15·4	13·4	13·6
10	M.	14	24·9	14·5	22·9	11·0
Average			25·9	23·4	20·6	23·6

Results

The fasting total blood cholesterol in the 10 normal individuals investigated varied from 133.3 to 195.3 mg./100 ml., the average being 160.3 mg./100 ml. After a cholesterol-rich breakfast there was observed in all cases a definite rise in the cholesterol curve which reached its maximum after five hours and returned almost to fasting level after seven hours. The increase was mainly due to an increase in free cholesterol. The ester level remained almost constant throughout the experiment.

Estimation of blood taken three hours after the standard breakfast showed in most cases a rise in the total cholesterol, but in a few cases values lower than the

fasting ones were observed. It is possible that the immediate result of a meal is an increased excretion and consequent lowering of the blood cholesterol, which is later corrected by absorption from the intestine. If this absorption is delayed the resulting lowering of the blood cholesterol is prolonged. This explanation is supported by the finding that the total blood cholesterol after five hours reached a maximum level in all cases.

The total blood cholesterol seven hours after ingestion of the standard breakfast was equal to, above, or below the fasting level. This can be explained (Bloor, 1943) by the fact that cholesterol is readily synthesized as required, and that any excess is normally excreted. Thus, after five hours it reaches a maximum in the blood, and any excess is excreted, either in the bile or, as coprosterol, by the intestine.

In the case of the five normal subjects whose blood cholesterol was estimated also three hours after taking the standard lunch, the total blood cholesterol was found to be lower than after the five hours level. It is probable that sufficient time had not elapsed for the postprandial increase to occur.

The total blood cholesterol showed another maximum level four and a half hours after lunch similar to that found five hours after breakfast.

Summary

Contrary to the statements of various workers that the changes in the blood cholesterol after a single meal are variable, the present workers have found a definite cholesterol tolerance curve in normal individuals which can be obtained after the ingestion of a meal rich in cholesterol. This curve shows the following characteristics: (1) a maximum level for total blood cholesterol five hours after ingestion; (2) a return almost to fasting level seven hours after ingestion; (3) the increase is mainly due to an increase in the free cholesterol, since the level of the esters remained nearly the same throughout the experiment.

References

Bloor, W. R. (1943). *Biochemistry of the fatty acids*, p. 313. New York.
Bruger, M., and Somach, I. (1932). *J. biol. Chem.*, **97**, 23.
Burns, F. J. (1948). *R.I. med. J.*, **31**, 103.
Gardner, J. A. (1932). *Brit. med. J.*, **2**, 392.
—— and Gainsborough, H. (1928). *Biochem. J.*, **22**, 1048.
McEachern, J. M., and Gilmour, C. R. (1932). *Canad. med. Ass. J.*, **26**, 30.
McClure, C. W., and Huntsinger, M. E. (1928). *J. biol. Chem.*, **76**, 1.
Mjassnikow, A. L. (1926). *Z. klin. Med.*, **103**, 767.
Nepveux, F., and Uhry, P. (1936). "Glands endocrines et Nutrition," 10536, p. 1. Paris. In *Encyclopédie médico chirurgicale*, ed. Laffont, A., and Durieux.

J. clin. Path. (1952), 5, 208.

TECHNICAL METHODS

A MICRO-METHOD FOR BLOOD SALICYLATE ESTIMATIONS

BY

D. G. MOSS

From Withington Hospital, Manchester

(RECEIVED FOR PUBLICATION OCTOBER 19, 1951)

In the treatment of rheumatic fever by the administration of massive doses of salicylates, the importance of maintaining an optimum blood level of the drug has been generally recognized. The therapeutic level, however, is very close to the toxic level, and, since different patients receiving the same dosage of salicylates may have widely differing plasma levels, repeated blood estimations may be of great value, particularly during the first few days of treatment.

The methods available for the estimation, though numerous, have certain inherent drawbacks in their routine use. The chief of these is the comparatively large volume of serum or plasma usually required. Also, most methods give appreciable values when applied to the blood of patients not receiving salicylates, due to the presence of interfering substances in the blood. This may entail the application of an approximate correction, by the use of a calibration curve constructed from pooled normal blood serum or plasma. Finally, many methods are not simple or rapid enough for routine use by technicians in small laboratories.

The methods which have been most commonly employed are based on the violet colours formed when ferric salts are added to salicylate solutions. Keller (1947) and Peters (1947) used direct addition of acidified ferric nitrate to serum or plasma, but Brodie, Udenfriend, and Coburn (1944) extracted the plasma with ethylene dichloride, and then returned the salicylates to an aqueous phase as a coloured ferric complex. A micro-method based on the same colour reaction has recently been published by Miller and Whitehead (1949), but a special technique is required.

The colour reaction of Jorrisen, obtained when salicylate solutions are boiled with copper sulphate and nitrous acid, was utilized by Mallick and Rehmann (1945) and by Reid (1948) for the estimation of blood salicylates, while Volterra and Jacobs (1947) first nitrated the salicylates and then estimated the nitro derivatives colorimetrically in alkaline solution. Weichselbaum and Shapiro (1945) and Smith and Talbot (1950) have used the blue colour which results when a salicylate solution is mixed with Folin and Ciocalteau's phenol reagent and made alkaline with sodium carbonate.

Diazo reactions have been widely employed for the estimation of *p*-aminosalicylic acid in blood, but do not appear to have been used for the determination of salicylic acid itself. This, no doubt, is due to the comparative slowness of coupling diazo compounds with salicylates. The difficulty, however, can be overcome by using a

large excess of the diazo reagent under carefully controlled conditions. The present method is based on coupling with diazotized *p*-nitraniline to give an orange dye, and was evolved to fill what was felt to be a need for a method which would (1) give blanks on normal blood which would be sufficiently low to be negligible by comparison with the salicylate levels commonly encountered during treatment, (2) be applicable to 0.2 ml. of whole blood collected by finger prick, and (3) be sufficiently rapid and simple for routine use in small laboratories.

In carrying out the estimation on whole blood, laking with water followed by precipitation of the proteins by trichloracetic acid was tried initially, but this procedure was found to lead to high blank values on normal blood, due presumably to liberation of interfering substances from the red cells. Dilution of the blood with isotonic sodium sulphate and protein precipitation by zinc hydroxide, as used by King (1946), gave more satisfactory results, and this technique has been adopted. Filtration, rather than centrifugation, has been preferred, as it was found that, in the latter case, small floating fragments of protein precipitate were occasionally captured by the pipette and caused increased colour. Since only traces of salicylates are present in the red cells (Smith, Gleason, Stoll, and Ogorzalek, 1946 ; Lester, Lolli, and Greenberg, 1946), whole blood levels are lower than the corresponding plasma levels. The former, however, can be converted to the latter, to a close approximation, by use of the equation : $p = b/100–h$, where p and b are the plasma and whole-blood levels in mg./100 ml. and h is the haematocrit. Table II shows a comparison of plasma levels calculated in this way and the observed levels. An exception was found in a fatal case of aspirin poisoning, in which a considerable concentration of salicylate was present in the red cells.

Method

Reagents.—The following are required : (1) Isotonic sodium sulphate (2% $Na_2SO_4.10H_2O$; (2) 10% zinc sulphate ; (3) 0.5N sodium hydroxide ; (4) 1.5% *p*-nitraniline in 5N hydrochloric acid ; (5) 10% sodium nitrite (stored in the ice-chest, this keeps for several months) ; (6) 4N sodium hydroxide ; (7) standard salicylate solution (50 mg. sodium salicylate dissolved in 100 ml. water. Store in the ice-chest).

Procedure.—Serum, plasma, or whole blood, 0.2 ml., are mixed with 6.6 ml. of isotonic sodium sulphate. Then 0.6 ml. of zinc sulphate solution followed by 0.6 ml. of 0.5N sodium hydroxide are added to precipitate the proteins, and the mixture is filtered. A Whatman No. 1 paper is suitable. A standard and a reagent blank are carried through simultaneously with the unknown by substituting 0.2 ml. of salicylate solution and 0.2 ml. of isotonic sodium sulphate for the blood, and 4 ml. of filtrate are mixed with 0.8 ml. of *p*-nitraniline solution. The solution is chilled in ice water. Sodium nitrite solution, 0.6 ml., is added, and, after standing in the ice water for two or three minutes, the solution is made alkaline by adding 1.2 ml. of 4N sodium hydroxide. The colours develop immediately and are stable for a considerable time.

Fig. 1 shows the light absorption of the reagent blank and salicylate standard respectively. Since the diazo reagent is present in large excess, subtraction of these two curves gives the absorption spectrum of the azo dye formed in the reaction. The wavelength of maximum light absorption of the latter compound is 500 mμ. If a spectrophotometer is used for the estimation, this wavelength setting should therefore be used. Alternatively an Ilford spectrum blue-green filter (No. 603) may be used. For routine use we have found a Chance blue filter (No. OB 2), used in conjunction with a Hilger " Biochem " absorptiometer, to give satisfactory results.

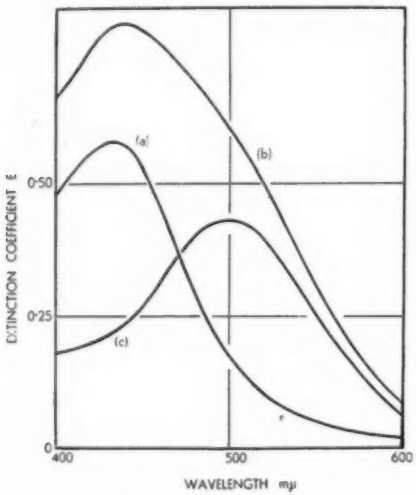


FIG. 1

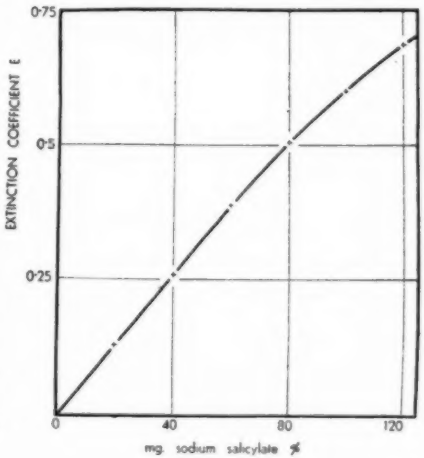


FIG. 2

FIG. 1.—Absorption spectra of (a) reagent blank, (b) salicylate standard, (c) salicylate azo compound, obtained by subtracting (a) from (b). Light absorption was measured on a "Unicam" spectrophotometer.

FIG. 2.—Calibration curve obtained by carrying out the analysis on salicylate solutions of the concentrations shown. Extinction coefficients were measured on a Hilger "Biochem" absorptiometer.

Thirty-five blood specimens from normal persons, not receiving salicylates, were examined by the present method. The estimations on whole blood gave values ranging from 0.0 to 3.0 mg./100 ml., with an arithmetical mean of 1.5 mg./100 ml. The range for plasma was 1.5 to 3.5 mg./100 ml., with a mean of 2.0 mg./100 ml.

Fig. 2 shows a typical calibration curve. A linear relationship between colour intensity and salicylate concentration was observed up to a concentration of 70 mg./100 ml. If levels in excess of this figure are encountered, they may be found by reference to the calibration curve, or alternatively the estimation may be repeated on a smaller volume of

TABLE I

RECOVERY OF SODIUM SALICYLATE FROM WHOLE BLOOD AND PLASMA

Added (μg.)	Whole Blood		Plasma	
	Found (μg.)	Recovery (%)	Found (μg.)	Recovery (%)
50	43	86	41	82
60	53	88	54	90
70	64	91	62	89
80	73	91	68	85
90	87	90	81	90
100	87	87	88	88

Mean recovery from whole blood 89%. Mean recovery from plasma 86%.

TABLE II

BLOOD SALICYLATE LEVELS

Plasma Salicylate Level* (mg. %)	Whole Blood Salicylate Level† (mg. %)	Haematocrit	Plasma Level Calculated from Whole Blood Level (mg. %)	Plasma Level (Smith and Talbot Method mg. %)
12·5	9·0	36	14·0	15·0
13·5	9·0	42	15·5	16·5
21·0	14·0	40	23·5	19·0
22·5	15·5	31	22·5	19·5
22·5	13·5	41	23·0	22·5
23·0	16·5	37	26·0	21·5
76‡	61	48	117	86

* A blank of 2·0 mg. % has been subtracted. † A blank of 1·5 mg. % has been subtracted. ‡ A fatal case of aspirin poisoning.

filtrate, the volume being corrected to 4 ml. with distilled water. The recoveries of sodium salicylate added to plasma and whole blood are shown in Table I.

A series of estimations on blood from patients receiving sodium salicylate is given in Table II. Simultaneously with the present method, the plasma salicylate level was determined by Smith and Talbot's method. Except for one exceptional case the greatest difference found was 3 mg. %.

I should like to thank Mr. H. Varley, M.Sc., of the clinical laboratories, Manchester Royal Infirmary, for the use of a spectrophotometer, and to Dr. W. Edgar for assistance in the collection of blood specimens.

REFERENCES

Brodie, B. B., Udenfriend, S., and Coburn, A. F. (1944). *J. Pharmacol.*, **80**, 114.
Folin, O., and Ciocalteu, V. (1927). *J. biol. Chem.*, **73**, 627.
Keller, W. (1947). *Amer. J. clin. Path.*, **17**, 415.
King, E. J. (1946). *Micro-Analysis in Medical Biochemistry*. London.
Lester, D., Lolli, G., and Greenberg, L. A. (1946). *J. Pharmacol.*, **87**, 329.
Mallick, S. M. K., and Rehmann, A. (1945). *Ann. Biochem. exp. Med.*, **5**, 97.
Miller, J. H., and Whitehead, R. W. (1949). *Rocky Mtn med. J.*, **46**, 544.
Peters, J. T. (1947). *Acta med. scand.*, **129**, 299.
Reid, J. (1948). *Quart. J. Med.*, n.s. **17**, 139.
Smith, M. J. H., and Talbot, J. M. (1950). *Brit. J. exp. Path.*, **31**, 65.
Smith, P. K., Gleason, H. L., Stoll, C. G., and Ogorzalek, S. (1946). *J. Pharmacol.*, **87**, 237.
Volterra, M., and Jacobs, M. D. (1947). *J. Lab. clin. Med.*, **32**, 1282.
Weichselbaum, T. E., and Shapiro, I. M. (1945). *Amer. J. clin. Path.*, **15** (Tech. sect., 9), 42.

J. clin. Path. (1952), **5**, 212.

ORTHOTOLIDINE HYDROCHLORIDE TEST FOR BLOOD IN URINE

BY

H. ZWARENSTEIN

From the Department of Physiology and Pharmacology, University of Capetown

(RECEIVED FOR PUBLICATION NOVEMBER 20, 1951)

The procedure previously described (Zwarenstein, 1949) has been modified as follows.

A small knife-point (about 5 mg.) of solid *o*-tolidine hydrochloride is added to 5 drops of urine in a test-tube and thoroughly mixed. A drop of the mixture is placed on a small piece of Whatman No. 1 filter paper (about 17 mm. square) on a white porcelain tile. The drop is allowed to soak into the paper and one drop of acid-peroxide reagent is added.

Negative and positive reactions for haematuria are the same as those described previously. Free haemoglobin, however, gives a coarse granular reaction. When tests for sensitivity were performed with the new procedure it was found that positive reactions at the higher dilutions were much easier to read and smaller amounts of red cells and free haemoglobin could be detected. Slight but definite positive reactions were obtained with a urine containing 1,000 red cells per ml. (a dilution of 1 in 5 million) and with a urine containing 50 μg. free haemoglobin per 100 ml. (a dilution of 1 in 300,000). The results were more consistent than with the original procedure.

It must be emphasized that the 3% hydrogen peroxide used for the preparation of the acid-peroxide reagent must be freshly diluted from 30% and that the mixture of equal volumes of glacial acetic acid and 3% hydrogen peroxide should be allowed to stand for 24 hours before using.

REFERENCE

Zwarenstein, H. (1949). *Journal of Clinical Pathology*, **2**, 145.

FOR CHEMICALS TO SPECIFICATION

The Analytical Control Laboratories at Dagenham

Insist on M&B

Laboratory control by our own analysts forms the basis of our service to the user of M&B* Laboratory Chemicals and Reagents. Up-to-date facilities for this work enable us to offer a range which maintains consistently high standards of purity in accordance with rigid specifications. These specifications, which are clearly set out on the containers, have been selected to meet a wide variety of laboratory procedures.

Over 500 different chemicals are available for prompt despatch from pre-packed stocks. Presentation in specially designed containers ensures maximum protection at all times.

* *trade mark*

M&B *brand* LABORATORY CHEMICALS AND REAGENTS

manufactured by
MAY & BAKER LTD · DAGENHAM · ENGLAND
Tel. ILFord 3060 *Ext.* 40

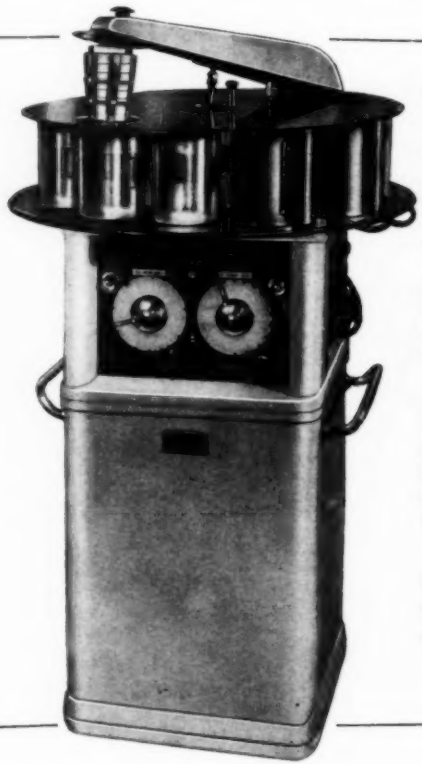
LA27

OF BRITISH DESIGN AND MANUFACTURE

THE *Elliott*

AUTOMATIC

TISSUE PROCESSOR



FEATURES :—

- Simplicity in Handling.
- Clean Design.
- Robust Construction.
- Flexibility.
- Delayed Start Mechanism (for 48 Hour Treatment)
- Alarm Circuit to protect Specimens.

SAVES :—

BED HOURS

By enabling processing to be carried on over night and weekends.

MAN-POWER

By freeing the Technician of slow routine.

WORRY

By doing its job without supervision and by guaranteeing comparable results.

EXPENSE

By its low initial and running costs.

Brings you at LOWER COST the full advantages of AUTOMATIC FIXATION—DEHYDRATION—INFILTRATION and STAINING OF HISTOLOGICAL SPECIMENS.

Further particulars upon application to the makers :—

Elliotts Liverpool Ltd.,

Scientific Instrument Makers. Laboratory Furnishers.

351, AIGBURTH RD., LIVERPOOL, 17. Phone : LARK LANE 1172.

J. clin. Path. (1952), **5**, 213.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 48th GENERAL MEETING

The forty-eighth general meeting of the Association of Clinical Pathologists was held in Portsmouth from April 3 to 5, 1952.

Summaries of the papers read at the scientific sessions follow.

Professor D. S. Russell, M.D., D.Sc., LL.D., F.R.C.P., took the chair at the first session.

Pre-juvenile Amaurotic Idiocy

Dr. H. S. BAAR reported on the histopathological findings in the extremely rare pre-juvenile or late infantile form of amaurotic idiocy. The illness started at the age of 4 years and the child died when 10 years old. A brief summary of clinical findings was given. The main post-mortem findings were atrophy of the brain which affected equally cerebrum and cerebellum, patchy chronic leptomeningitis, laminar spongy devastation mainly in layers 3 to 5, disappearance of nerve cells, inflation of remaining nerve cells by a granular material which took most of the lipid stains and was insoluble in alcohol, ether, chloroform, and benzene. The granules stained blackish-brown with Weigert's haematoxylin, but not by the Smith-Dietrich method. With the former stain some nerve cells appeared to be replaced by mulberry-like clumps of granules. The variations in staining properties of different nerve cell types and of compound granule cells and in their behaviour in polarized light were stressed. The nerve cell changes were ubiquitous but mildest in the spinal cord, severest in pyramidal and Purkinje cells. Swelling and abnormal ramification were most marked in apical dendrites of Purkinje cells, where a club-like swelling filled with lipid granules was sometimes larger than the cell-body. Gliosis of grey matter was diffuse, but varied in density. Intracellular neurofibrils were pushed to the periphery or absent. Axons were sometimes swollen but did not contain lipids; their neurofibrils appeared to undergo granular disintegration. Demyelinization was severe at the hilum of the dentate nucleus of cerebellum, marked in the cerebral cortex and in the immediately subcortical white matter, mild in deeper layers. Some genetic aspects were discussed.

Circulatory Changes in the Liver in the Newborn

Dr. J. L. EMERY said that the left physiological lobe of the liver in the newborn occasionally appeared deep red and slightly shrunken. The left and right lobes of the liver of 100 newborn children were examined histologically. Approximately one in three cases of children dying in the first two days of life, one in six in the first fortnight, and one in 10 in the first two months show a relative shrinking in the cells of the left lobe. This asymmetrical change was not seen in the stillborn children. The incidence of alteration in the left lobe of the liver followed that of patency of the ductus venosus. The lesion may be due to a temporary shunting of the blood from the left portal vein through the ductus venosus.

Diagnostic Value of Dudgeon's Rapid Smear Technique

Dr. GIBSON emphasized the importance of speed in transferring the smears to the fixative because drying destroys the nuclear pattern. Lantern slides of photomicrographs of the smears were shown to illustrate the appearances of benign and malignant cells.

At the first session on April 4 the chairman was Dr. E. M. Darmady.

The Value of the Rib Biopsy in Marrow Disorders

Drs. M. S. R. HUTT, P. SMITH, and J. L. PINNIGER reported that rib resection had been found to be a valuable method of obtaining bone marrow for histological examination during life. The procedure had been used in 12 patients and had always yielded excellent marrow sections. The operation was simple and no serious post-operative complications had been encountered.

Further Observations on the Vitamin B_{12} Concentrations in the Serum and Urine of Patients with Megaloblastic Anaemias

Drs. D. L. MOLLIN and G. I. M. ROSS reported *Euglena* assays of vitamin B_{12} in serum of 57 patients with megaloblastic anaemias treated with B_{12}, folic and folinic acid. The average duration of raised concentrations after B_{12} injections appeared proportional to the amount retained. Urinary excretion of B_{12} by these patients and normal subjects was similar after injections.

Atypical Congenital Haemolytic Anaemia

Drs. J. V. DACIE, P. L. MOLLISON, J. G. SELWYN, and NANCY RICHARDSON reported that of the surprisingly large number of different types of congenital haemolytic anaemia, the best known were hereditary spherocytosis, Mediterranean anaemia, and sickle cell anaemia. Other types existed and six less well-known types were mentioned.

Twelve families in whom atypical cases had occurred were studied. The disorder was classified as non-spherocytic in type in five families; as a hypochromic type distinct from Mediterranean anaemia in one family; as variants of hereditary spherocytosis in two families; as a macrocytic type with leg ulceration in one case; as variants of familial elliptocytosis in two families; and as a type associated with thrombocytopenia and "triangular" red cells in one case.

The case histories of six patients were referred to and their very varied blood pictures illustrated by photomicrographs. The results of osmotic and mechanical fragility tests and of studies of the rates of autohaemolysis were also given.

The effect of splenectomy was variable; in four patients of the non-spherocytic type the operation was a failure, and this was also true of the patient belonging to the macrocytic type and of the patient with thrombocytopenia and "triangular" red cells. In patients with variants of hereditary spherocytosis and familial elliptocytosis respectively splenectomy was definitely beneficial.

The chairman at the second session on that day was the President, Sir Lionel Whitby, C.V.O., M.C., M.D., F.R.C.P.

Compatibility Tests Preceding Transfusion

Dr. P. L. MOLLISON said that a compatibility test must ensure, first, that there will be no rapid destruction of transfused red cells by iso-antibodies. This may be described as the immediate risk of incompatibility and, in practice, it lies almost entirely in transfusing blood of the wrong ABO group. However, in a special group of recipients, namely those who have had previous transfusions or women who have had pregnancies, there is a definite risk of trouble from antigens outside the ABO system and, above all, from the Rh antigen (D). Secondly, a compatibility test must ensure that there is no likelihood that the transfusion will sensitize the patient to a blood group antigen so that a subsequent transfusion or pregnancy provokes antibody formation. In practice this risk lies almost entirely in transfusing Rh-positive blood to an Rh-negative subject.

A suitable matching test is a protection only against the immediate risks of transfusion. The following technique is recommended.

A 2% suspension of the donor's red cells in 30% albumin is made, and one small volume added to an equal volume of the recipient's serum in a tube. The mixture is left for a minimum of 10 minutes; the red cells are spun down, then the deposit is examined under the low power of a microscope. This test is better than any slide test, since it is not possible in a slide test to provide optimal conditions for detecting both weak agglutinins and weak incomplete antibodies.

The tube test in albumin should be supplemented by the indirect Coombs test in recipients who may have been previously sensitized by transfusions or pregnancies.

To guard against sensitizing recipients to foreign antigens it is sufficient to ensure that Rh-positive blood is not transfused to Rh-negative subjects. Whenever time permits, the Rh-group as well as the ABO group both of donor and recipient should be determined before transfusion. If no tests can be done all women must be assumed to be Rh negative until proved to the contrary.

Errors in the Cross-matching Test

Dr. F. STRATTON, Manchester, spoke on the subject of errors in the cross-matching test. These were divided into two varieties: false positive and false negative. False positive ones consisted of the well-known trio of rouleau formation, cold agglutination, and infection, together with certain clotting difficulties.

A recognition of rouleau depended first upon its microscopical appearance, which in saline agglutination is distinctive and recognizable, and secondly upon the fact that the patient's serum in a final dilution of 1:3 loses its rouleau-forming properties. In certain cases, such as multiple myelomatosis, rouleau may be very marked.

Cold agglutination is due to true agglutination present only at room temperature and not at 37° C.

False positive results due to infection present as an agglutination at room temperature but not at 37° C. Here, the purple appearance of the cells and the smell of the blood sample may assist in the diagnosis. It is noted that the control tube containing patient's own cells and patient's own serum is valuable in diagnosing false positive errors and in distinguishing one from another.

False negative results are due to zoning, especially in the albumin test, and to haemolysins in the case of anti-A and anti-B antibodies.

An insensitive technique is a major cause of such error, and the tube technique was recommended as the only one suitable for use in the cross-matching test. The albumin test was considered to be the one that must be used if a single test only could be employed, but the difficulties of the albumin technique were enumerated and it was suggested that, by preference, a saline agglutination test between the donor's cells and the recipient's serum should be carried out at room temperature and 37° C. and a Coombs test at 37° C. should also be performed.

Blood Transfusion during 1951 in a Hospital Group of 700 Beds

Dr. G. H. TOVEY said that a survey of the use of blood, plasma, and dextran in a hospital group of 700 beds during 1951 showed that 1,039 patients were transfused with a total of 2,463 pints of blood, 110 pints of plasma, and 169 pints of dextran. The ratios of pints of blood transfused to number of beds per main department were: medical, 4.5:1; surgical, 5.8:1; obstetrical, 2.5:1; gynaecological (principally abortions), 7.8:1; paediatric, 1.1:1; general practitioner, 0.9:1. Adverse reactions to the fluids transfused were few, the only adverse reactions to dextran being a rigor in one patient and urticaria in another. Both patients had received blood in addition to dextran. Restoration of blood volume was satisfactorily accomplished by dextran transfusion.

A major problem was the excessive amount of blood requested to be matched and held in the blood bank for patients undergoing operation. On an average 28 pints of blood were matched unnecessarily each week from this practice, and a detailed analysis showed that in a limited number of major surgical procedures only was blood transfused with a frequency greater than 30%. Blood was now matched only for patients undergoing more extensive operations, or if the patient had a history suggesting that iso-immune antibodies might be present in the serum. Otherwise the patient's ABO and D-group were determined pre-operatively, but no matched blood was held.

The afternoon was occupied by demonstrations, and for the third session Dr. R. W. Fairbrother, D.Sc., F.R.C.P., took the chair.

Electrolytes and *in vitro* Bacteriostatic Activity

Dr. B. W. LACEY said that the effect of replacing sodium chloride by any of 30 salts or salt mixtures on the sensitivity of eight organisms to six bacteriostatic agents had been tested by a filter paper disc method on a medium containing agar, starch, glycerol, alanine, glutamate, cysteine, salts equivalent to 0.7% sodium chloride, and 33% horse blood. The salts were the chlorides, sulphates, acetates, malonates and aconitates of lithium, sodium, magnesium, and ammonium. The organisms were all normal or pathological pharyngeal flora: a diphtheroid, *Staph. pyogenes*, a penicillin-resistant *Staph. pyogenes*, *Strep. faecalis*, a paracolon, *H. influenzae*, *H. parapertussis*, and *H. pertussis*. The bacteriostats were bacitracin, penicillin, aureomycin, streptomycin, chloramphenicol, and 4:4 diamidino-diphenylamine (M and B 938). The susceptibility of bacteria and bacteriostatic agents to changes in the ionic composition of the medium increases in the order of listing above. The effect of a given ion varies both with organism and inhibitor. With chloramphenicol and *H. pertussis* at least, the effect of a cation may also vary with the anion. At times the effect of a given ion on bacteriostatic activity largely reflects the action of the ion on the growth of the organism. Thus lithium markedly diminishes the growth and increases the sensitivity of *Strep. faecalis* to all bacteriostatic agents. At others the ionic effect may be independent of any obvious effect on growth. Thus sulphate markedly decreases the sensitivity of most organisms to M and B 938 without any change in colony size. The differential effect of ions on the sensitivity of different organisms had been exploited in the development of a selective medium for *H. pertussis*. Media with different salt content from those in use at present might be found to give a better *in vitro* estimate of the therapeutic value of some substances, especially perhaps of chloramphenicol and diamidines.

The Selective Distribution of Certain Antibiotics in Animal Organs

Dr. J. UNGAR said that, since Florey *et al.* (1946) had shown that penicillin could be detected in wound exudates for a considerably longer period than in blood, laboratory evidence had substantiated this finding. The distribution of injected penicillin esters, of streptomycin, and of aureomycin in normal and inflamed tissue, particularly in infected lungs, had also been investigated. It was seen that penicillin salts were retained in any inflamed tissue, whereas penicillin esters were concentrated to a higher degree in inflamed lung tissue only. Streptomycin was also retained in inflamed tissue, and aureomycin, in addition, was concentrated in inflamed lungs and even more so in the normal liver.

The distribution of the injected antibiotic in the body organs depends on the drug's physical and chemical properties and on the physiological activities of those organs. There is strong evidence that alteration in the chemical composition of the inflamed tissue plays a part in the retention of an antibiotic and that the concentration of enzymes (esterases) and of nucleic acids is significantly altered in inflamed tissues. It was further noted that antibiotics displayed varied pharmacodynamic actions, depending on their chemical constitution, and that these would influence the distribution of the antibiotic and the selective concentrations in certain organs.

Observations on Specific Serological Types of *Bact. coli* in Children

Dr. G. T. STEWART reported that between November, 1951, and February, 1952, 250 cases of non-specific enteritis in young children were investigated. Specific *Bact. coli* was isolated from only 17 (6.8%), a figure which did not differ significantly from the figures for strains of *Proteus* and paracolon bacilli in the same group. The numbers of isolations for specific *Bact. coli*, as for *Proteus* and the paracolon bacilli, were significantly higher in children under 2 years of age than in older children. A significantly higher proportion (30%) of isolations of specific *Bact. coli* was made from a group of 40 symptomless children, of comparable age groups,

who were contacts of some of the cases of enteritis from whom the specific strains had been recovered.

Analysis of the strains recently isolated showed that type 055 (Aberdeen β) was most common. In 1950 and the earlier part of 1951 this strain was uncommon in the Paddington-Marylebone area.

Two of the most severe cases observed in the series, both fatal, were described. One patient died with haemorrhagic pneumonic lesions from which *Bact. coli* type 026 was isolated; the other died with signs of septicaemia, *Bact. coli* type 055 being isolated from the blood.

It was concluded that specific strains of *Bact. coli* were not at present commonly associated with enteritis in London children. Older children, and contacts of cases, might harbour large numbers of the organisms without disease, but the potential pathogenicity of some strains, in children under 2 years of age, was very high, and not necessarily limited to the gastro-intestinal tract.

The Incidence and Demonstration of Biological Reactions in Routine Wassermann Reactions

Dr. H. M. RICE said that many non-syphilitic, positive Wassermann reactions could be distinguished by the Harrison-Wyler-Lacey method (dilute-antigen row added to the standard test). Sensitivity and specificity were both increased. With the dilute antigen, weak sera showed less, and biological sera more, haemolysis than with the standard suspension. The results of 15,870 consecutive routine Wassermann reactions, including minimal reactions normally disregarded, showed 263 frank biological reactions (1.66%), 69 positives (0.43%) and 194 doubtfuls (1.22%). Females showed 43 positives (0.57%) and 119 doubtfuls (1.50%) in 7,479 tests (total 162 = 2.16%); males showed 26 positives (0.31%) and 75 doubtfuls (0.89%) in 8,391 (101 = 1.20%). Hospital cases gave: female, total 71/5,228 (1.36%); male, total 42/4,401 (0.95%); V.D. clinics 91/2,251 female (4.04%) and 59/3,990 male (1.43%).

Some biological reactions were masked, i.e. of syphilitic reaction type. To obtain comparative figures all positive and doubtful reactions were analysed, and final diagnoses determined. Results of syphilis cases were excluded, though sero-positive cases may sometimes show biological reactions. Total biological reactions, including repeat tests, were 325/15,870 (2.05%); positive 93 (0.59%), doubtful 232 (1.46%).

Some doubtfuls were only detectable by the dilute antigen test, being negative to the standard tests. Detailed analyses were shown. Frank biologicals comprised the following percentages of all biological reactions: female, V.D., positive 100, doubtful 95; hospital, positive 56, doubtful 82; male, V.D., positive 59, doubtful 79; hospital, positive 69, doubtful 75; overall total for series 81. Simple dilute antigen techniques were, therefore, most useful for distinguishing biological from syphilitic reactions, in addition to increasing both sensitivity and specificity.

On April 5 the chairman was Dr. E. N. Allott, F.R.C.P.

The Level of Plasma Amino-acids and Phenols in Liver and Renal Disease

Drs. E. M. DARMADY, J. A. DURANT, J. HARKNESS, and PATRICIA A. LOUD reported that in studying acute renal failure with uraemia it soon became clear that the blood urea did not accurately indicate the degree of toxicity, and in view of the success of the high calorie diet which was stated to prevent the endogenous breakdown of protein and the overproduction of amino-acids, it was surprising to find that a number of cases showed no increase in amino-acids either in total amount or in abnormal distribution.

An investigation was therefore made into 78 cases, 36 of which showed kidney failure and/or uraemia. As a result it was found that although the blood urea was raised, the level of phenols was more in keeping with the symptoms of uraemia. On the other hand, in studying the toxicity of liver failure the phenol levels were also frequently found to be normal but the total of amino-acids was raised. Before undertaking this work the normal levels of both amino-acids and phenols were investigated in 30 normal cases. These ranged from 0.9 to 2.5 phenols and 4.2 to 7.2 amino-acids.

Single Injection Methods for Renal Clearance Tests

Dr. C. P. STEWART described formulae developed in his laboratory (Robson, Ferguson, Olbrich, and Stewart, 1950) which permitted the measurement of inulin, diodone, or *p*-amino-hippuric acid clearances after a single intravenous injection of a carefully measured amount of the test substance and without catheter collection of urine. After the injection two samples of blood are required and, at the same (widely spaced) times, two samples of urine with complete emptying of the bladder. The urine samples are required to measure the amount of test substance excreted up to the time of their collection; the second one also gives the volume of urine excreted during the test period (Olbrich, Ferguson, Robson, and Stewart, 1950).

The formulae avoid many of the fallacies of previous single injection methods, since they do not assume equilibration between plasma and extravascular fluids. Rapid and violent fluctuations of water distribution might interfere, since the formulae give "average clearance" over 60 to 100 minutes, but in practice this is not a serious objection. Nor, because of the long test period and the slow change in plasma concentration, is any serious error introduced because of slow mixing in the collecting tubules. The use of venous blood for the analyses, for substances which, like diodone, show a considerable arteriovenous difference in concentration, involves the development of a new normal range. For inulin clearance the new normal is the same as for the infusion method, for diodone clearance it is 20% lower, and for Tm_D 8% lower. With this correction the formulae provide clearance figures with as great accuracy as is given by clearance methods, and with a very considerable simplification of technique as well as a reduction in the analytical work.

REFERENCES

Olbrich, O., Ferguson, M. H., Robson, J. S., and Stewart, C. P. (1950). *Lancet*, **2**, 565.

Robson, J. S., Ferguson, M. H., Olbrich, O., and Stewart, C. P. (1950). *Quart. J. exp. Physiol.*, **35**, 111, 173.

The Comparison of Radioactive and Metabolic Methods of Thyroid Investigation

Drs. J. B. FOOTE, D. H. MACKENZIE, and N. F. MACLAGAN reported that the degree of correlation between the basal metabolism and the thigh-neck clearance (Foote and Maclagan, 1951) had been examined in 162 cases mostly suffering from various disorders of the thyroid gland. One hundred and forty of these were untreated and 22 had received treatment with various drugs. There was a fairly close correlation (correlation coefficient = 0.76 ± 0.035) in the 140 untreated cases, but those treated with iodine, thiouracil, or thyroid extract showed a gross discrepancy between the two tests and fell well outside the main correlation area. These deviations are summarized in the following table.

FORMS OF TREATMENT CAUSING LACK OF CORRELATION BETWEEN BASAL METABOLISM AND THIGH-NECK CLEARANCE

Therapy	B.M.R.	Thigh-Neck Clearance
Goitrogen	N or L	H
Thyroid	N or H	L
Iodine	H or N	L

The high degree of correlation between the two tests in untreated cases may be taken as confirmation of the value of the thigh-neck clearance in the assessment of thyroid activity. In treated cases the radioactive test is of limited value.

REFERENCE

Foote, J. B., and Maclagan, N. F. (1951). *Lancet*, **1**, 868.

The Nature of Complement

Professor J. R. MARRACK said that Heidelberger (1941) showed that antigen-antibody precipitates formed in the presence of fresh guinea-pig serum contained more N than precipitates formed in presence of saline or of heat inactivated serum. He inferred that this extra N was the N of complement. Similar amounts of N are added to antigen-antibody precipitates from fresh human, ox, and pig serum. These sera contain widely different amounts of complement and of components of complement, measured by ability to promote lysis of sheep red cells sensitized by horse or rabbit antiserum.

Recently Johns measured the N in precipitates formed by constant antibody with varying amounts of antigen in presence of active and heated sera. The N added (1) is very small with heated human sera; (2) is about the same per ml. with fresh human and horse sera as had been found with guinea-pig and pig sera; (3) with heated horse serum, varies with the amount added and the amount of antigen and may increase or decrease the precipitate N.

Conglutinin is contained in serum of oxen and other ruminants; it is heat stable. It promotes agglutination of particulate antigens treated with antibody and with fresh serum of horses and some other species, supplying conglutinating complement. Conglutinating and haemolytic complements seem to differ in their fourth components. Johns finds that ox serum adds further N to precipitates formed in the presence of both fresh and heated human serum, but not in the presence of horse serum.

Fresh serum appears to contain some protein, the amount of which varies little among the species studied but varies much in ability to promote haemolysis or conglutination in the presence of ox serum.

REFERENCE

Heidelberger, M. (1941). *J. exp. Med.*, **73**, 681.

The final session was presided over by Dr. L. R. Jones.

The Effect of Cortisone Therapy on the Histology of Rheumatoid Arthritic Joints

Drs. D. KING and D. WILSON said that five patients suffering from rheumatoid arthritis were selected for this investigation. Three patients were treated with daily intramuscular cortisone, and biopsies of affected knee-joints were performed before the start of treatment, at eight weeks, and at 15 weeks. The remaining two patients served as controls. Dr. Wilson described the clinical features and briefly detailed the clinico-pathological investigations and reactions. The increasing delay in healing of the successive biopsy wounds during treatment was a striking feature. Dr. King drew attention to the inherent difficulties of obtaining comparable biopsies, and demonstrated the variable inflammatory reaction in juxtaposed synovial membrane. He was able to show that after more than three months of treatment the inflammatory reaction was still active and did not reflect the marked clinical improvement.

Barbiturate Levels in Cases of Intoxication

Dr. L. C. NICKOLLS said that the clinical pathologist is often faced with cases of coma in patients received in hospital, which may be due to barbiturates. Rapid diagnosis is essential.

Barbiturates may be divided roughly into three categories on the basis of speed of action and behaviour in the body as follows:

Classification of Barbiturates	Speed of Action of Fatal Dose	Behaviour in Body
(1) *Fast:* amytal, seconal, soneryl, etc.	Thirty minutes to 10–15 hours	Absorbed from stomach. Destroyed in body.
(2) *Medium:* phenobarbitone and medinal	Few hours to three days	Stomach contains some barbiturate. Part destroyed. Part excreted.
(3) *Slow:* rutonal, barbitone	One to 6 days	Stomach usually contains gross amounts of drug. Circulates until excreted.

Cases of the first type do not often reach the hospital, since death supervenes so rapidly. The barbiturate level does not exceed 5 mg. per 100 ml. of blood or C.S.F. however great the amount taken, since death occurs as soon as this level is reached or exceeded. Moreover, if the amount taken was a minimum lethal dose so that death is unduly delayed, the body may contain no residual barbiturates at all.

Cases of the second type are commonly received while still comatose and with a good hope of recovery. The barbiturate level is a minimum of 5 mg. per 100 ml., and may rise to double this figure.

Cases of the third type are comparatively rare nowadays, but when they do occur it will be found that the blood barbiturate level is often very high. He had found as much as 25 mg. of barbitone per 100 ml. of blood still circulating several days after ingestion.

The levels given above indicate that with types 1 and 2 a sensitive method of identification is essential. The better modifications of the Cobalt test are sensitive to about 0.1–0.2 mg.

This means that a minimum of 5 ml. of blood are needed for a satisfactory test. In these circumstances it is obvious that a stomach washout is a more satisfactory sample, since even after some days there is still a minimum of several milligrammes of barbiturate present.

Necropsy Findings in Three Members of a Family

Dr. D. E. PRICE said that in 1951 the deaths of three young children, whose mother was a nurse, were investigated. The deaths occurred over a period of 22 months, and each was inquired into by H.M. coroner before they were considered together.

The first to die was the second child at the age of 4 months. A "general practitioner" finding at necropsy was "bronchopneumonia." The third child died 18 months later when it was 6 months old, a few hours after an operation, as an out-patient, for removal of a naevus from the vulva. A pathologist diagnosed at necropsy "acute heart failure following operation for excision of a naevus with status thymolymphaticus contributing." The cause of death in the case of the eldest child, the last to die, was "barbitone poisoning," and the circumstances suggested that the child had either consumed the tablets herself in mistake for sweets or had been fed with the tablets by another person.

The records showed that barbitone poisoning could not be excluded in the two earlier cases and that on each occasion the only person who had access to the child was the mother.

Exhumation was carried out. The findings were described. There was no evidence of poisoning by barbitone drugs. The findings of the previous inquiries were allowed to stand.

The attention of the meeting was drawn to the following points: (1) the negative character of the findings at post-mortem examinations in cases of barbitone poisoning; (2) the vast use of the barbitone group of drugs and the careless attitude adopted towards them; (3) the danger of post-mortem examinations being conducted by doctors with no forensic pathology included in their training.

Disposal of the Body by Burying

Dr. H. S. HOLDEN dealt with two cases in which considerable ingenuity was shown in the disposal of the body of the victim following murder.

In the first of these the body was interred under the brick floor of the coal cellar. To avert suspicion the murderer advertised the victim as missing in both the local press and a widely circulated Sunday paper. He also secured a B.B.C. announcement asking for her return. Intensive police investigation following a study of the "missing persons" file resulted in the discovery of the body 12 months later.

In the second case the murderer confessed the crime committed nine years previously. He had put the body in a sack and buried it in the earth below a large heap of farmyard manure in a field adjacent to a cow byre. Although the site of the dung heap had been cleared long before, he indicated to the investigating officers the actual place of burial and the remains were disinterred and examined in detail.

In both cases the description of the persistent fragments of clothing and the study of the dentures which were recovered proved of material help in identification.

J. clin. Path. (1952), **5**, 221.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the two abstracting journals, Abstracts of World Medicine, and Abstracts of World Surgery, Obstetrics and Gynaecology, published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Nocardamin, a New Antibiotic from a Species of *Nocardia*. STOLL, A., BRACK, A., and RENZ, J. (1951). *Schweiz. Z. allg. Path.*, **14**, 225.

From the culture fluid of a *Nocardia* sp. the authors isolated an antibiotic which appears to be quite distinct from those obtained from other *Nocardia* spp. It has been isolated in crystalline form and its chemical constitution is considered to be $C_9H_{17}O_2N_3$. It is inactive against Gram-positive and Gram-negative cocci as well as against Gram-negative bacilli, even in a dilution of 1 in 1,000. It is, however, actively bacteriostatic against strains of human, bovine, and avian tubercle bacilli, as well as against *Mycobacterium phlei* and *Myco. smegmatis, in vitro* in dilutions varying from 1 in 1,600 to 1 in 64,000.

G. M. Findlay.

Pertussis: Clinical Comparison of the Newer Antibiotics. BOOHER, C. E., FARRELL, J. B., and WEST, E. J. (1951). *J. Pediat.*, **38**, 411.

Streptomycin (28 cases) was found to be of little use in the treatment of pertussis.

Discussing their results, the authors point out that there is little difference in the efficacy of chloramphenicol, aureomycin, and terramycin, but state that aureomycin has the disadvantage of producing gastric disturbance with accompanying loss of weight. It is not thought that any specific for pertussis has at present been discovered.

Streptomycin Resistance in Strains of Tubercle Bacilli coming from Different Pulmonary Lesions in the Same Patient. CANETTI, G., and SAENZ, A. (1951). *Ann. Inst. Pasteur*, **80**, 238.

A series of lungs from 24 persons with pulmonary tuberculosis who had died after receiving streptomycin were examined to determine the sensitivity of the tubercle bacilli isolated from different lesions. In 8 cases the strains isolated from the different lesions of the same patient had the same degree of sensitivity; in 5 cases tubercle bacilli with slightly different degrees of sensitivity were isolated, but the differences were not statistically significant. In 11 patients tubercle bacilli of very different sensitivity were isolated, and in 7 cases some quite sensitive bacilli were isolated and from different lesions bacilli which were not inactivated by 1,000 g. per ml. In 4 patients some bacilli were feebly resistant and others highly resistant. The best chances of isolating bacilli of different sensitivity are in patients who have not been treated for long periods.

The frequency and the degree of resistance of the strains of *Mycobacterium tuberculosis* isolated are directly related to the content of the lesions in bacilli. Thus, lesions yielding less than 100 colonies gave rise to 48.4% of sensitive strains, 25.8% of moderately resistant strains, and 25.8% of highly resistant strains. If the lesions yielded more than 1,000 colonies the figures were 10.9% of sensitive strains, 21.7% of moderately resistant strains, and 67.4% of highly resistant strains. Lesions with soft casein yielded resistant strains more frequently than did lesions with solid casein. This difference is usually due to the fact that soft casein lesions contain more tubercle bacilli, but possibly the nature of the lesions plays some part apart from the number of bacilli present.

G. M. Findlay.

Chloromycetin in the Treatment of Bacillary Urinary Infections. WILSON, W. M., BARNES, R. W., and BERGMAN, R. T. (1951). *Urol. cutan. Rev.*, **55**, 206.

The authors selected 21 cases of urinary infection to test the effect of chloramphenicol. The cases were chosen according to the following criteria: (1) Gram-negative bacillary infection; (2) previous unsuccessful treatment with another chemotherapeutic agent; (3) no demonstrable organ lesion.

The routine course given to the patients was: 750 mg. four times a day for 3 days, then 250 mg. (1 capsule) four times daily for 6 days. Of the 21 cases 19 cleared up after the first course of chloramphenicol; in 9 cases infection recurred after cessation of the drug; 5 of these patients were found to have some definite pathology accounting for this persistent infection.

The findings suggest that *Proteus* and paracolon bacillus infections are not as sensitive to chloramphenicol as are other Gram-negative organisms.

F. B. Cockett.

Culture of Tubercle Bacilli. Comparison between Cultures of Laryngeal Swabs with Cultures of Gastric Washings. GILJE, A. (1951). *Nord. Med.*, **45**, 129.

For his comparison of cultures of tubercle bacilli the author took laryngeal swabs (20 to 30 at a time) and gastric washings from the same patient. The swabs were examined in the laboratory of the sanatorium and the washings were sent, after neutralization, to Oslo, where 5·5% of 618 gastric washings and 14·8% of 863 laryngeal swabs from the same patients were found to be positive. Of 53 patients who were either sputum-negative (40) or had no sputum (13), positive cultures were obtained from gastric washings alone in 2, from laryngeal swabs alone in 35, and from both washings and swabs in 12. Thus, the laryngeal swabs were positive in 51 and the gastric washings in 14; if the gastric washings had been tested alone, there would have been 35 false negative results, and if the laryngeal swabs had been used alone, only 2 false negatives among the 53 known positives.

Margaret Agerholm.

Identification of *Staphylococcus pyogenes* by the Phosphatase Reaction. BARBER, M., and KUPER, S. W. A. (1951). *J. Path. Bact.*, **63**, 65.

The phosphatase reaction was used to detect *Staphylococcus pyogenes* in routine cultures. Nasal swabs taken from 100 persons were plated on nutrient agar containing phenolphthalein diphosphoric acid in a concentration of 0·01 g. per 100 ml., and after incubation at 37° C. for 18 hours the presence of free phenolphthalein in the colonies, indicating phosphatase activity, was detected by exposing the plates to ammonia vapour. Colonies showing the reaction were found on 42 plates; the appearance observed is illustrated in coloured photographs. Phosphatase-negative and phosphatase-positive colonies were inoculated separately into tubes of broth and tested for coagulase production after incubation at 37° C. for from 6 to 24 hours. No coagulase activity was found in colonies taken from the 58 phosphatase-negative plates. Of 85 phosphatase-positive colonies 84 were coagulase-positive, and of 75 colonies giving negative or doubtful results in the phosphatase tests 70 were coagulase-negative. The phosphatase reaction could also be produced in medium containing 5% horse blood, although the pink colour was less marked, and was not affected by sodium chloride in a concentration of 5%.

The results show that colonies which give a negative result in the phosphatase test may be inferred to be coagulase-negative, and that a positive phosphatase reaction is a good indication of coagulase production if the technique is followed carefully.

D. J. Bauer.

BIOCHEMISTRY

Significance of Raised Serum-enzyme Levels in Perforated Peptic Ulcer. WAPSHAW, H. (1951). *Brit. med. J.*, **1**, 995.

The serum amylase level is sometimes raised in patients with perforated peptic ulcer. Investigating the cause in dogs, Pemberton *et al.* (*Proc. Mayo Clin.*, 1950, **25**, 5) found that the serum amylase level was slightly raised when experimental perforation occurred 1 to 2 hours after a meal, but was not raised when 3 to 4 hours had elapsed between the meal and perforation. Examination of the escaped fluid in the peritoneal cavity in the two groups showed that the amylase and lipase content in the first group was six times that of the second.

The author studied the serum amylase and lipase levels in 30 patients with perforated peptic ulcer (29 pyloro-duodenal, 2 gastric, 1 anastomotic), and found the serum amylase level to be raised (above 200 units per ml.) in 6, the highest levels occurring when the perforations had been present for a long time (15 and 24 hours) without repair. The serum amylase level returned to normal 24 hours after repair except in one case. The serum lipase level was raised in 4 patients. There was a wide variation in the amylase content of the fluid that had escaped into the peritoneal cavity (from *nil* to 32,000 units) which did not correspond with the serum amylase level, in contrast to the findings of Pemberton *et al.* in dogs. The peritoneal fluid lipase content varied from 0.1 to 14.3 ml. of N/20 NaOH. There was no correlation between the serum amylase level and the time which had elapsed between eating

and perforation; the highest concentrations were found in patients who had not eaten for several hours before perforation occurred.

The reason for the raised serum amylase and lipase levels following perforation in man is not understood. Two possibilities are suggested: (1) peritoneal absorption of fluid containing enzymes that had escaped from the alimentary tract; and (2) pancreatitis caused by the irritating nature of the escaped fluid. Inactivation of the enzymes by acid gastric juice and the stimulus to secretion of the previous meal are two factors which may confuse the chemical investigation.

Charles P. Nicholas.

The Interrelationship of Blood and Urine Diastase during Transient Acute Pancreatitis. DANKNER, A., and HEIFETZ, C. J. (1951). *Gastroenterology*, **18**, 207.

The quantity of diastase and creatinine present in the blood and excreted in the urine was measured at intervals of 1 to 2 hours during 13 attacks of transient acute pancreatitis occurring in 12 patients. The urinary excretion of diastase paralleled the blood diastase values throughout the attack. During a period of normal renal function high initial blood diastase values were followed in less than 2 hours by increased urinary excretion of diastase. Examination of the figures for creatinine concentration showed that acute pancreatitis, even in the absence of clinical shock, frequently impaired renal function, the effect being greatest 24 to 48 hours after the start of the attack and lasting for from 12 to 36 hours. When renal impairment occurred the blood diastase values returned to normal more slowly. If the renal damage occurred early the initial increase in urinary diastase values was delayed. *J. E. Page.*

The Value of Blood Pyruvic Acid Estimations during the Performance of the Glucose Tolerance Test. GENNES, L. DE, BONFILS, S., and DELTOUR, G. (1951). *Pr. méd.*, **59**, 497.

Simultaneous blood glucose and blood pyruvate estimations were performed at ½-hourly intervals after administration of 50 g. of glucose to fasting subjects. Concentration of pyruvate in the blood was roughly 1/100 that of the glucose. In normal subjects the peak blood glucose level was at 30 minutes, but the peak blood puruvate level was at about 60 minutes. Both returned to normal in 2 hours. In diabetics the rise in blood pyruvate level was either small or absent, while in cases of obesity it was also smaller than normal and might be lacking. In alcoholic cirrhosis there was a delayed but steady rise in pyruvate level, maximal at 90 to 120 minutes. In cases of Cushing's syndrome the rise tended to be higher and to be more sustained.

The significance and value of the blood pyruvate curve are discussed. It is suggested that the test may be able to detect incipient diabetes before this is revealed by the blood sugar curve. *C. L. Cope.*

HAEMATOLOGY

Chronic Involvement of the Liver in Intestinal Amoebiasis (Chronic Amoebic Hepatitis). SANDLER, R. (1951). *Amer. J. digest. Dis.*, **18**, 29.

In contrast to acute amoebic hepatitis and hepatic abscess, chronic amoebic hepatitis has hitherto attracted little attention. The present author has, however, found symptoms which he attributes to chronic hepatic involvement in 47 of 180 cases of amoebiasis seen in southern Israel during the last 4 years. These symptoms, which persisted uninterruptedly for months, included anorexia, nausea and vomiting, diarrhoea or constipation, flatulence, tenderness and often slight enlargement of the liver, and occasionally pain in the right hypochondrium, shoulder, or back, felt only in certain postures.

Entamoeba histolytica was found in the stools in 34 of the 47 cases; the complement-fixation test was positive in 20 out of 25. Liver function tests generally gave normal results, although the formalin test was positive in 5 cases out of 6. Emetine was of no diagnostic or therapeutic value. The hepatitis appeared gradually to subside following routine treatment of the intestinal infection. Most of the cases were followed up "for years," and in none did liver abscess subsequently develop.

H. McC. Giles.

Successful Blood Transfusion by Means of a Siliconelined Syringe in a Case of Haemorrhagic Thrombocytopathia. FAVRE-GILLY, J., HIRSCH, I., DAMESHEK, W., and PRATT, J. (1950). *Sang*, **21**, 681.

A child of 9 years had since birth had a haemorrhagic diathesis which was not relieved by splenectomy. The bleeding time was prolonged and the platelets present were morphologically abnormal though not unduly reduced in number. The prothrombin index was

normal, but the prothrombin conversion by autologous platelets was defective. The bleeding tendency was not affected by a transfusion of citrated blood. The transfusion of blood from a thrombocythaemic donor by means of silicone-coated syringes was successful in arresting the haemorrhage: it was possible to show survival of morphologically normal platelets for several days with a reduction in bleeding time and prothrombin conversion. *John F. Loutit.*

Coagulation Defects with Intrauterine Death from Rh Isosensitization. WEINER, A. E., REID, D. E., ROBY, C. C., and DIAMOND, L. K. (1950). *Amer. J. Obstet. Gynec.*, **60**, 1015.

Defects of blood coagulation have been noted occasionally in cases of obstetrical complications. The authors, at Boston Lying-in Hospital, have observed 4 cases of Rh sensitization in which the foetus was known to have been dead *in utero* for several weeks and in which these coagulation defects were present. In 12 similar cases observed during a recent 2-year period no abnormality of the clotting mechanism was noted.

The first patient had skin ecchymoses, and the subsequent clinical course was complicated by intra-partum and post-partum haemorrhage. It was therefore decided to study the blood coagulation mechanism in subsequent cases. The second patient began to bleed after delivery of a foetus which had been dead *in utero* for 10 weeks, and she continued to do so in spite of a well-contracted uterus and administration of oxytocics. Blood transfusion to a total of 6,000 ml. was given, together with vitamin C and vitamin K, but in spite of this the blood failed to clot; later it was found that the clot dissolved within an hour of its formation. After the administration of 3,000 mg. of fibrinogen, blood loss was notably diminished but still continued, and subtotal hysterectomy was performed. Subsequently the patient's condition gradually returned to normal.

In the third patient, intrauterine death of the foetus occurred at the sixth month, and 2 months later ecchymosis and vaginal bleeding were observed. Clotting time was 15 minutes and plasma fibrinogen concentration was 35 mg. per 100 ml. After delivery 500 ml. of unclotted blood escaped, but intravenous administration of fibrinogen restored the clotting mechanism to normal within 24 hours. No subsequent abnormality was found.

In the fourth case the foetus died at the sixth month, at which time the plasma fibrinogen concentration was 313 mg. per 100 ml. Two months later it had fallen to 50 mg. per 100 ml. and the following day to 35 mg. At this point bleeding began and 2,000 mg. of fibrinogen was administered, this dose being repeated twice in 12 hours. One hour after delivery the plasma fibrinogen level had risen to 230 mg. per 100 ml. and clotting time was normal.

Thus in 3 out of 15 patients a transient afibrinogenaemia occurred following intrauterine death of the foetus resulting from Rh isoimmunization. The syndrome can be recognized, and fibrinogen replacement is effective in correcting the defect.

[Fibrinogen does not appear to be generally available for clinical use in Britain, but the occurrence of this syndrome in 20% of a series of observed cases suggests that it is important that stocks should be held by all blood banks.]

J. A. Chalmers.

MORBID ANATOMY AND HISTOLOGY

Papillary Cystadenoma Lymphomatosum of the Parotid Gland. HOLMBERG, R. E., and GLOVER, D. M. (1951). *Surgery*, **29**, 240.

The authors have collected 151 cases of papillary cystadenoma from the literature and added 8 further cases of their own. These tumours arise 6 times more often in men than in women and occur later in life, most commonly in the sixth decade. The usual site is superficial, at the angle of the jaw or in the tail of the gland. They should be treated by total local excision and, when so treated, are unlikely to recur. The pathological characteristics are described and the various theories of origin, both old and new, are discussed.

[Nicholson's view that these tumours arise from heterotopic salivary tissue in lymph nodes, probably the most commonly accepted opinion in Great Britain, is not mentioned.]

A. J. Drew.

An Embryological Tumor of Retinal Anlage Involving the Skull. CLARKE, B. E., and PARSONS, H. (1951). *Cancer*, **4**, 78.

A tumour of the skull in the neighbourhood of the anterior fontanelle in a male infant was found microscopically to consist of cells of two types, some neuroblastic, others epithelium-like and pigmented. The diagnosis of "pigmented retinoblastoma" was made [in the abstracter's opinion, on inadequate grounds].

R. A. Willis.

J. clin. Path. (1952), **5**, 225.

PRIMARY EPITHELIAL TUMOURS OF THE JAW

BY

T. F. HEWER

From the Department of Pathology, University of Bristol

(RECEIVED FOR PUBLICATION MARCH 3, 1952)

Primary epithelial tumours in the jaw are an interesting group in that they may be derived from the lining epithelium of the mouth or from that which has invaded the jaw during the embryonic development of the teeth. The exact origin of these tumours is often debatable, their diagnosis radiologically and histologically not always easy, and their prognosis therefore in doubt.

The morphological possibilities of epithelial tumours of the jaw can only be appreciated if the details of the embryology of the teeth are borne in mind. It will be remembered that the ectodermal lining of the mouth grows into the primitive gum in the form of a solid dental lamina in the seventh week of development. At intervals along this lamina thickenings of the epithelium appear and constitute the enamel organs. At a later stage, as in the 16 weeks' embryo illustrated in Fig .1, the enamel organ comes to lie over the mesenchymal dental papilla and has a central core of so-called stellate reticulum, which is a further differentiation of the epithelium, and a marginal layer of columnar enamel-forming ameloblasts. Ordinarily the stellate reticulum, the outer layer of ameloblasts, and the dental lamina disappear by the time the deciduous and permanent teeth are formed, but remnants of this epithelium may occasionally remain and give rise to neoplastic growth at a later date.

It is important to remember that the adult oral mucosa, composed of squamous epithelium, of ectodermal or entodermal origin, has a capacity at least as great as that of the epithelium of the body surface to produce the whole range of epithelial tumours: simple inclusion and retention cysts, squamous cell carcinoma, basal cell tumours of "rodent" type, and adamantinomas. Any of these may invade the jaw and on occasion it is difficult to decide whether the tumour arose from an intra-alveolar site or from the mucous surface.

Most tumours in the jaw declare their presence by causing a swelling, and on radiological examination an area of absorption of bone is generally detected. It is not the purpose of this paper to discuss the diagnostic radiological features. Our experience in the Bristol Bone Tumour Register has been that one cannot always rely upon radiological appearances, and unfortunately a histological examination of a biopsy specimen may be misleading. Taken together the two methods generally enable one to make an accurate diagnosis, but only if the limitations of both are borne in mind.

In a study over a period of six years of 10 examples of simple dental cysts, 12 adamantinomas, and five intra-alveolar carcinomas, and various carcinomas of the mouth cavity invading the jaw, we have been impressed particularly by the frequency with which a wrong clinical diagnosis has been made and by the hazards of histological diagnosis on small biopsy specimens. A few examples of these different types of epithelial tumour, some benign and some malignant, will serve to illustrate these features.

Benign Epithelial Cysts

Simple periodontal cysts may be derived from epithelial cell nests (of Mallassez) in the area of the periodontal membrane, or from the remnants of the deep part of the developmental dental lamina. They are lined by a very simple squamous epithelium which is often damaged by secondary infection of the fluid or keratinous contents. Toller (1948) has shown that the hydrostatic pressure within these cysts may be as much as 70 cm. of water, so it is not surprising that they cause pressure atrophy of the bone in which they are embedded and occasionally become quite large.

A periodontal cyst may also result if the original infection in a periapical granuloma clears up and leaves a cavity which becomes lined by squamous epithelium and contains epithelial debris and cholesterol crystals. There is generally a dense fibrous wall with evidence of chronic inflammation.

Another simple tumour occurring in the jaw is a dentigerous cyst, of which Fig. 2 is an example. This arises alongside a deciduous tooth and encloses the permanent tooth below it: it is lined by squamous epithelium and can be recognized only by its characteristic relationship to the teeth.

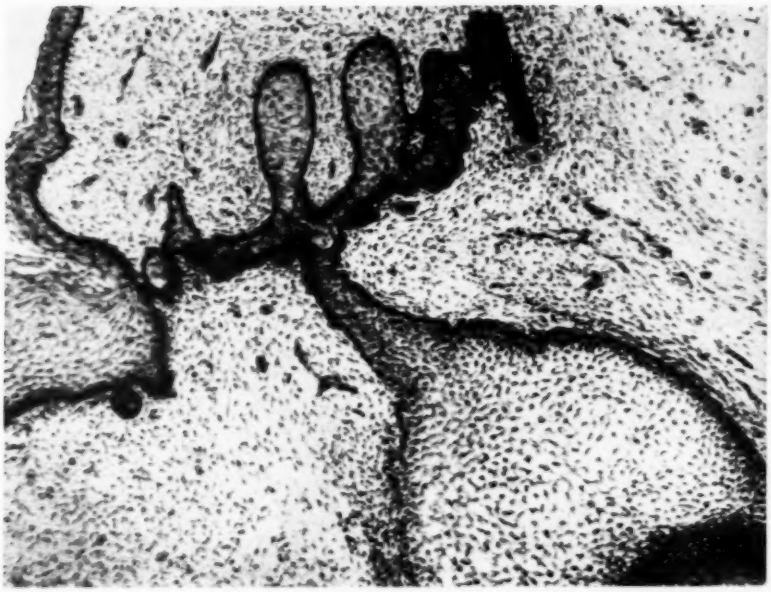


FIG. 1.—Deciduous tooth germ of 16 weeks embryo. The oral mucosa is on the left. The dental lamina arising from it contains two masses of squamous epithelium outlined by cubical basal cells. The enamel organ, descending from the lamina, has a central core of stellate epithelial "reticulum" with an outer limiting layer of columnar ameloblasts. The concave base of the enamel organ is a cellular mass of larger ameloblasts lying on the mesenchyme of the tooth germ. × 150.

All of these benign cysts are unilocular and have a clear-cut outline radiographically. Compound odontomes are congenital malformations, or else result from inflammation of the tooth germ, and we need not consider them here.

FIG. 2.—The dental cyst, arising in association with the root of the deciduous tooth, encloses the crown of the permanent tooth below it.

Malignant Epithelial Tumours

It is with the invasive or malignant tumours that the chief histological difficulty is encountered because the biopsy specimen may have a deceptively simple appearance. The commonest tumour in this group is the adamantinoma which is generally supposed to arise from the outer layer of ameloblasts of the enamel organ and to be locally invasive. It is characterized histologically by rounded or elongated islands of epithelium with a well-defined margin of columnar cells, similar to those of the enamel organ, and a central area of stellate reticulum which is also epithelial and may contain large or small cystic spaces. They are commonly multilocular and have a "soap-bubble" appearance radiographically, but unfortunately this is not an entirely reliable criterion for diagnosis.

It must be remembered that histologically similar tumours may be found elsewhere in the oral, nasal, and pharyngeal cavities; in the region of the pituitary as components of a craniopharyngioma; and in the tibia and other bones. And areas resembling an adamantinoma are occasionally seen in a squamous carcinoma of the surface skin, so one cannot limit their origin to the enamel organ.

A few details of seven selected cases of malignant epithelial tumours of the jaw will serve to illustrate some of the diagnostic pitfalls.

Case Reports

Case 1 (B.T.R. 129).—In 1937 a woman aged 35 had an operation for evacuation of a "dentigerous cyst" of the mandible. Next year there was a local recurrence and the condition was described as an "odontoma": the cavity was scraped out. In 1942 she was thought to have fibrocystic disease of the jaw and a cyst was removed and the bony walls of the cavity crushed inwards. In 1945 the cyst recurred and a fourth conservative operation was performed.

On November 24, 1947, the left ramus was found expanded and a radiograph showed the characteristic "soap-bubble" appearance of an adamantinoma. The left half of the mandible was completely removed and the patient has remained free of recurrence.

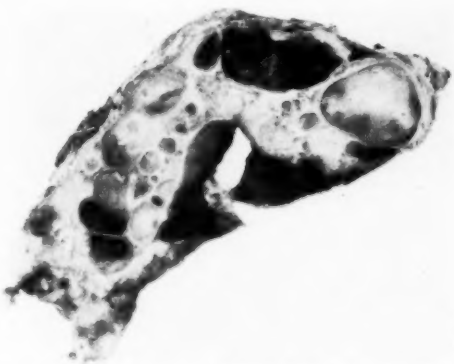


FIG. 3.—Mandibular ramus completely filled with adamantinomatous cysts.

The specimen (Fig. 3) consisted of an expanded mandibular ramus with numerous, smooth-lined cysts containing glairy fluid or, in some cases, haemorrhagic contents. Histologically (Fig. 4) the cystic spaces were due to degeneration of the stellate reticulum which, in places, was undergoing squamous metaplasia.

At this stage there was no possibility of mistaking the *x*-ray or the histological appearance.

Case 2 (B.T.R. 180).—In 1922 a woman aged 22 developed a swelling in the left side of the mandible. A number of small local operations were performed during the next 20 years with no histological examination. In 1942 a more radical local removal was done, but by 1949 the swelling recurred once more and the *x*-ray appearance was characteristic of adamantinoma absorbing a large part of the jaw. In March, 1949, the whole of the left half of the mandible was removed. In August, 1951, she was very well and declined a bone graft because with a lower denture she could manage perfectly and showed very little deformity.

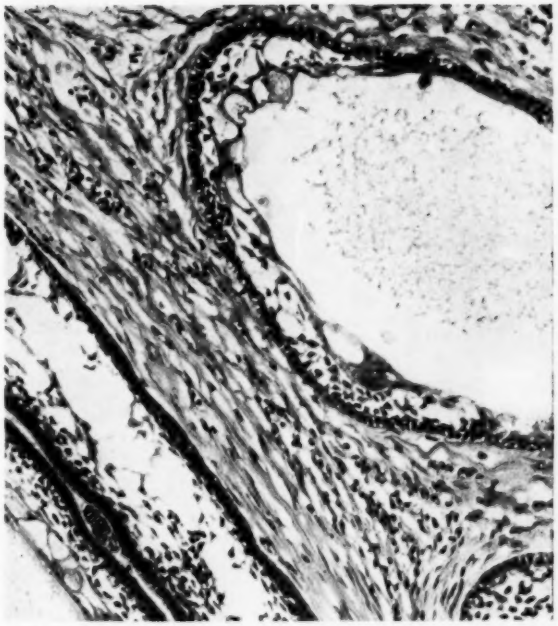


FIG. 4.—Section from the edge of one of the cysts in Fig. 3 showing characteristic degeneration of the stellate reticulum and marginal layers of columnar ameloblasts. The stellate cells lining the small cyst in the centre are showing some squamous differentiation, × 200.

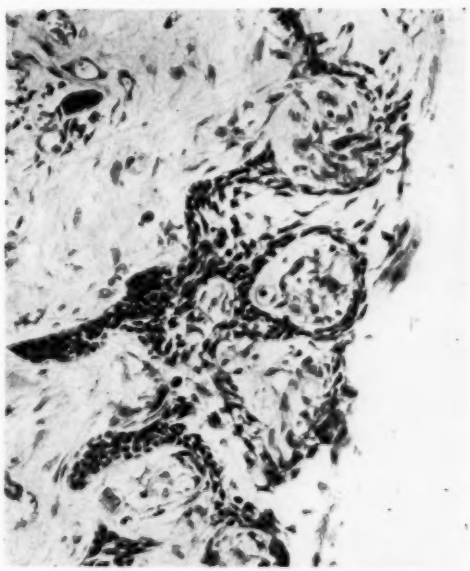


FIG. 5.—Degenerate lining of a large adamantinomatous cyst. Cubical basal cells are covering vascular fibrous projections and some ragged stellate epithelium is recognizable, × 200.

The specimen contained several small and two large cysts. The lining of the larger cysts (Fig. 5) was degenerate but still recognizable as adamantinoma. Papillary fibrous and vascular projections were covered by epithelium of rather basal-cell type, with fragments of stellate reticulum. Haemorrhage into the cysts had occurred from the vascular stroma. The small cysts were almost solid masses of adamantinoma with minute cysts resulting from the degeneration of the stellate reticulum. Fig. 6 shows also some squamous metaplasia of this reticulum.

Case 3 (B.T.R. 120).—In 1937 a woman aged 46 had a "dental cyst" removed from the left side of the mandible. It recurred in 1939, 1945, and 1947 after further local removals, no histological examination being made. In March, 1948, at the age of 58, most of the left half of the mandible was removed with success.

FIG. 6.—From the same section as Fig. 5. A clearer picture of an adamantinoma, with some squamous metaplasia of small cysts, × 200.

The specimen (Fig. 7) showed cystic dilatation and absorption of almost the whole of the ramus and part of the body. The cysts were lined by typical adamantinomatous tissue and the more exuberantly granular areas showed some squamous metaplasia of the stellate reticulum (Fig. 8). In the lower central part of this illustration the squamous epithelium looks like a poorly differentiated squamous carcinoma, and if a small biopsy had been taken it is possible that such a diagnosis might have been made. In fact the tumour behaved like an adamantinoma, invaded locally, and did not spread to lymph nodes.

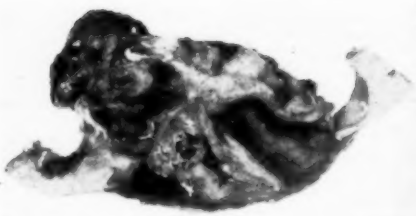


FIG. 7.—Ramus and large part of body of mandible occupied by adamantinomatous cysts.

Case 4 (B.T.R. 168).—In December, 1948, a man aged 58 was seen with a painless swelling of the right lower jaw of seven months' duration. A radiograph showed several cysts invading the jaw and a diagnosis of adamantinoma was made. The right half of the mandible was removed and replaced by a chondro-osseous graft from a rib. In August, 1951, there was no recurrence.

The specimen contained some five cysts, about 1 cm. in diameter, one extending into the condyle and another into the masseter muscle. They were lined with smooth, pearly-looking epithelium and contained friable keratinous material. Histologically (Fig. 9) there was a uniform lining of squamous epithelium with an orderly rather columnar layer at the base and no suggestion of alveolar arrangement, stellate reticulum, or any other evidence of adamantinoma; and yet it was behaving like an adamantinoma and had that gross and radiographic appearance. It seemed that this tumour must have been present for a very long time and produced no symptoms until it invaded the masseter muscle. The final histological appearance may be interpreted as a complete squamous metaplasia of an adamantinoma, although there is of course no proof that it ever had the characteristic adamantinomatous pattern. The important point is that if complete reliance had been placed upon its histological appearance the invasive nature of the growth would not have been suspected.

Case 5 (B.T.R. 323).—A woman aged 71 had had an ulcer for two years in an edentulous area of the right maxillary alveolus posteriorly. Radiologically there was absorption of the bony floor of the antrum, and a biopsy (Fig. 10) showed a basal cell carcinoma arising from the buccal mucosa. In April, 1951, the whole of the right maxilla was avulsed from the pterygoid plates and a stent mould covered with Thiersch graft was packed into the cavity. Further histological examination of the tumour showed a remarkable and gradual transition from a basal cell carcinoma to an alveolar epithelial tumour and, in the deeper part, to a perfectly characteristic adamantinoma (Fig. 11).

It is our opinion that this ulcer, which was never more than 1 cm. in diameter, was a basal cell carcinoma arising in the buccal mucosa and assumed the histological guise of an adamantinoma as it invaded the maxilla.

Case 6 (B.T.R. 399).—A woman aged 22 had a history of a progressive painful swelling of the right side of the mandible for eight months before she was seen and found, on *x*-ray examination, to have a cyst surrounding an unerupted third molar tooth. This was apparently a dentigerous cyst, but because of its rapid development and considerable extent, a biopsy was made before surgical treatment. This showed a characteristic adamantinomatous histological pattern, and in spite of the unilocular appearance it was treated as an adamantinoma, half the mandible being resected. On gross examination it was a dentigerous cyst, but in places the epithelial lining was coarsely granular and thicker than usual (Fig. 12). The less granular parts were lined by simple squamous epithelium with no invasion of the underlying bone, but in the thicker areas there were long pegs of epithelium invading deeply and becoming transformed into an adamantinoma (Fig. 13) which was invading the bone beyond the limits of the cyst. This case was interpreted as an example of a dentigerous cyst in which

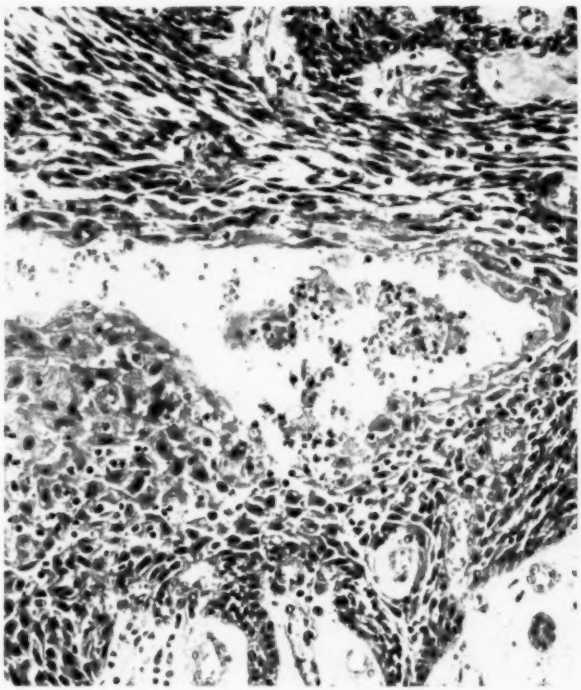


FIG. 8.—From the lining of one of the large cysts in Fig. 7. Note the considerable squamous metaplasia towards the lower right corner, resembling a squamous cell carcinoma. The rest of the picture is a pure adamantinoma. × 200.

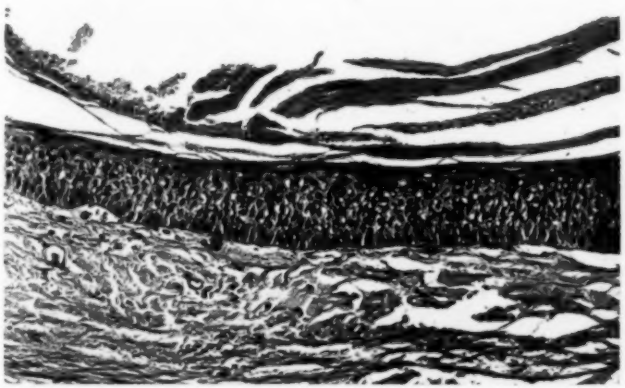


FIG. 9.—Wall of an adamantinomatous cyst of mandible with a uniform squamous epithelial lining, no stellate reticulum but inspissated keratinous contents. There is a columnar cell basal layer. × 200.

the squamous epithelial lining suffered an adamantinomatous change. If a biopsy had not been taken the cyst might well have been removed conservatively and the adamantinomatous invasion of the adjacent bone left behind.

Case 7 (B.T.R. 170).—In July, 1948, a woman aged 75 had her right lower third molar extracted because of severe toothache. The pain continued and she was treated with penicillin for osteomyelitis. A radiograph showed local bony rarefaction. By November, 1948, a swelling was visible in the mouth, but the mucosa was intact. A radiograph then showed extreme absorption of the bone of the ascending ramus and part of the body of the mandible approaching spontaneous fracture. A biopsy was performed and diagnosed angiosarcoma. In December, 1948, the whole of the right half of the mandible and the masseter muscle were removed; one of the upper cervical lymph nodes contained tumour, as did some at the angle of the jaw. Shortly after the operation the tumour recurred, did not respond to radiotherapy, and caused the death of the patient. The tumour (Fig. 14) was solid and pale grey, had destroyed a large part of the mandible, and diffusely invaded the adjacent muscles. There was ulceration of the overlying mucosa, but this had developed terminally. Histologically the tumour (Fig. 15) was a poorly differentiated, Grade 3 or 4, squamous carcinoma with a tendency to form alveoli. In some areas (Fig. 16) the alveolar pattern was dominant, but nowhere were there any columnar cells or any trace of stellate reticulum. It was not possible therefore to adduce an origin from the enamel organ, but all the clinical evidence was that it arose within the mandible, involving the overlying mucosa quite late, and this, together with the curious alveolar histological appearance, led us to call it an intra-alveolar squamous cell carcinoma.

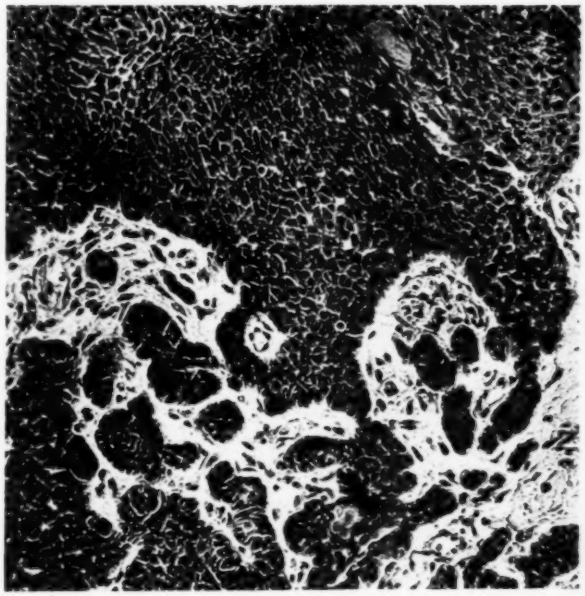


FIG. 10.—Basal cell carcinoma arising from oral mucosa on alveolus. Original biopsy. × 200.

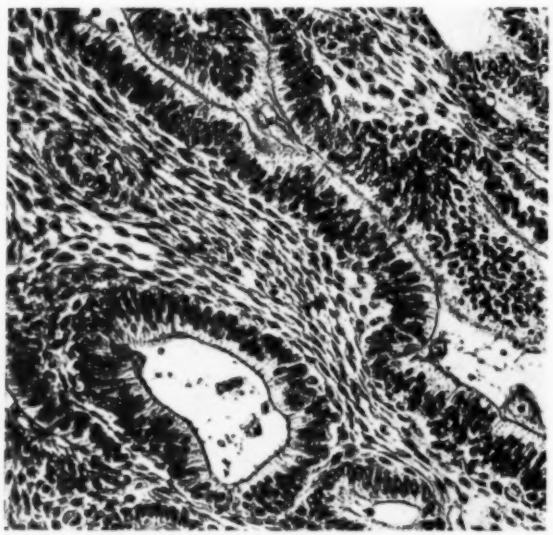


FIG. 11.—A deeper section after operation from the same case as in Fig. 10. The cystic spaces in this field are in the connective tissue stroma, not in the stellate reticulum which is almost solid. × 200.

Discussion and Conclusions

Cases 1, 2, and 3 are representative of many in which it was assumed originally that the lesion was a simple dental cyst and in consequence inadequate local operations were performed. If the true nature of these neoplasms had been appreciated early it would undoubtedly have been possible to operate adequately and avoid the necessity of ultimately removing half the mandible. So many of these cases have

FIG. 12.—Dentigerous cyst with adamantinoma arising in its wall and invading mandible.

FIG. 13.—Lining of cyst in Fig. 12 showing superficial squamous epithelium with adamantinomatous pegs passing deeply into the jaw. × 200.

come our way with a similar history of inadequate operations that we believe a histological study should be made of every dental cyst that is diagnosed, whether or not it is radiologically unilocular, and even if it is quite clearly a dentigerous cyst. Local crushing operations and conservative removal without histological control are dangerous procedures. When the clinical picture is very clearly that of a simple dental cyst it is not of course necessary to perform a biopsy before removal, but post-operative histological study should be the rule.

Since adamantinomas invade the bone very widely we have found it essential to examine the bone histologically at the lines of excision to make sure that the tumour has been eradicated with sufficient margin.

Unfortunately, as can be seen in Cases 4 and 5, it must be admitted that histological examination does not always permit an easy diagnosis to be made. Parts of an adamantinoma may undergo squamous metaplasia to such an extent that there may be a mistaken diagnosis of simple dental cyst. If a biopsy had been taken from the jaw in Case 4 the former might have happened: fortunately the *x*-ray appearance was so characteristically multilocular that radical operation was performed without any hesitation. On the other hand, if the squamous metaplasia is of an undifferentiated type it may be very difficult to distinguish the tumour from an intra-alveolar epidermoid carcinoma. Willis (1948) refers to this difficulty and prefers to call the whole group "carcinomas of the tooth-germ

FIG. 14.—Intra-alveolar carcinoma ulcerating into mouth and invading masseter muscle and local lymph nodes.

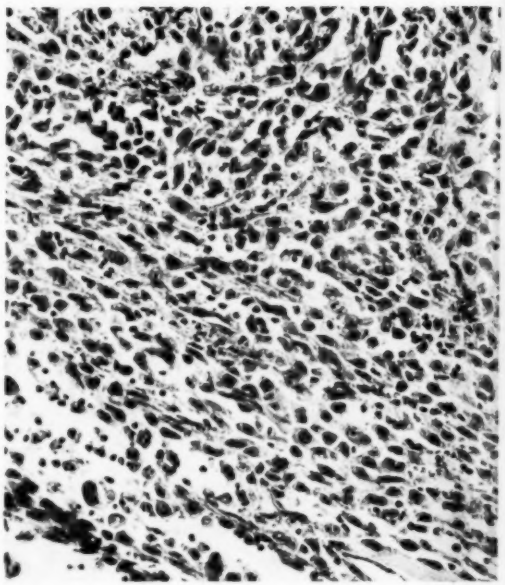


FIG. 15.—Same case as Fig. 14. Squamous carcinoma invading masseter muscle. × 200.

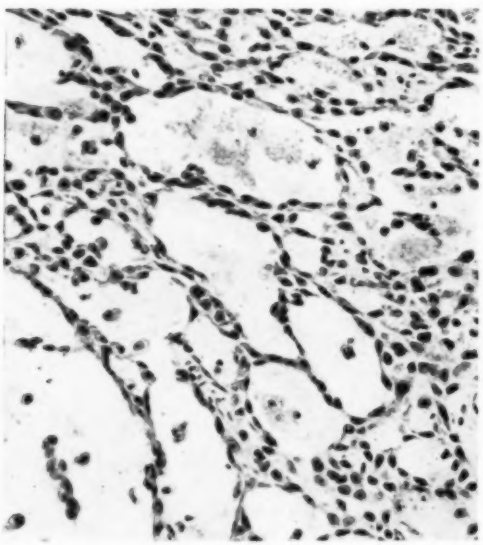


FIG. 16.—Intra-alveolar portion of same tumour as Fig. 15 showing alveolar pattern. × 200.

residues," but this diagnosis, without amplification, would be insufficient for the surgeon who wants to know whether the tumour is likely to remain within the jaw and grow slowly or invade the soft tissues and metastasize by lymph or blood stream. The intra-alveolar epidermoid carcinomas do spread to the lymph nodes and invade widely.

Everyone with experience of these tumours must have difficulty from time to time in knowing how to use the term "adamantinoma." My own view is that since the histological appearance of the typical example, with columnar cells and stellate reticulum, is so remarkable it is useful to apply the word "adamantinoma" to it; but it must always be remembered that this is not really a very specific label and that it does not necessarily mean that the tumour was derived from the enamel organ. I believe that Case 5 in this communication was an example of a basal cell carcinoma arising in the alveolar mucosa and invading the underlying maxilla in somewhat the same way as the embryonic mucosa invades the developing jaw to form a dental lamina. Basal cell carcinomas arising elsewhere do not show a particular tendency to adopt an adamantinomatous or alveolar pattern when they invade bone, and it would seem that there is something inherent in the oral mucosa which determines the frequency of adoption of that pattern in epithelial tumours of the jaw: it is not, of course, invariable, but it happens very often.

Carcinomas of tooth-germ residues might be expected on general morphological grounds to give

rise to tumours of columnar, basal cell type or to squamous epithelial variants: it is the variants that occasionally cause histological diagnostic difficulty. In Case 4 it seemed that complete squamous differentiation of an adamantinoma had occurred. It behaved like an adamantinoma and for that reason it seemed to me justifiable to call it an adamantinoma, because every surgeon will know what is meant by that term. For completeness it was only necessary to add that it had undergone squamous metaplasia without any evidence of the development of squamous cell carcinoma.

McWhirter (1950) suggests that rodent ulcers of the skin, adamantinomata, some salivary gland tumours, and bronchial adenoma should all be grouped together as "adnexal tumours," but this only introduces another not very helpful name and has no justification beyond his claim that they react similarly to radiotherapy. I would suggest that we can still use the terms "adamantinoma of the jaw, with characteristic histological pattern," "adamantinoma of the jaw, with squamous metaplasia," "adamantinoma of the maxillary antrum," "adamantinoma of the tibia," "basal cell carcinoma of the skin, with histological adamantinomatous pattern," and so on. There cannot be a single name for any of these things and there is little point in inventing new ones. It is enough to know that all the conditions mentioned above are locally and rather slowly invasive and do not ordinarily metastasize. By contrast, intra-alveolar epidermoid carcinoma invades widely and metastasizes to lymph nodes. The distinction between the two types is most important but occasionally difficult. One must, as in so many diagnostic problems, consider all the evidence available—clinical, radiological, and histological. Complete reliance on any one of these three means of diagnosis will sooner or later lead to error.

Summary

The origin and histological structure of epithelial tumours arising in or invading the jaw is discussed.

It is necessary to distinguish between simple epithelial cysts, adamantinomata, and intra-alveolar epidermoid carcinoma because of their differing prognostic significance.

Examples are presented to illustrate the difficulties in histological diagnosis of all three of these groups, and a plea is made for routine histological study in every clinical case of dental cyst, whatever the radiological appearance may be.

I am indebted to Mr. G. M. FitzGibbon, F.R.C.S., for providing me with most of my material and for the use of his records; to Dr. J. W. E. Snawdon for the use of the photograph of Fig. 2; to Dr. C. H. G. Price, Hon. Secretary of the Bristol Bone Tumour Register, for much of the histological material; and to Mr. G. H. Rogers for all the photography. The Bristol Bone Tumour Register is supported by a grant from the British Empire Cancer Campaign Committee.

REFERENCES

McWhirter, R. (1950). *J. Fac. Radiol., Lond.*, **2**, 31.
Toller, P. A. (1948). *Proc. roy. Soc. Med.*, **41**, 681.
Willis, R. A. (1948). *Pathology of Tumours*, p. 685. London.

J. clin. Path. (1952), **5**, 234.

LOWER NEPHRON NEPHROSIS AFTER TRANSURETHRAL RESECTION OF THE PROSTATE

BY

JOHN SWINNEY AND B. E. TOMLINSON

From the Departments of Urology and Pathology, Newcastle General Hospital

(RECEIVED FOR PUBLICATION MAY 28, 1952)

Oliguria and anuria due to lower nephron nephrosis may occur after crushing injuries (Bywaters and Dible, 1942), incompatible blood transfusion (Baker and Dodds, 1925; Foy, Altmann, Barnes, and Kondi, 1943), burns (Goodpastor, Levenson, Tagnon, Lund, and Taylor, 1946), sulphonamide therapy (Hellwig and Reed, 1942; Lederer and Rosenblatt, 1942), septic abortion (Bratton, 1941), concealed accidental haemorrhage (Young, 1942), and occasionally after operations, particularly the operation of transurethral prostatic resection. In the past three years we have observed six cases, which have followed prostatic resection out of a total of 742 such operations.

Creevy (1947) showed that when water is used as the irrigating fluid during prostatic resection, haemoglobinaemia occurs, due to absorption through the prostatic veins of lysed blood and water itself. Haemoglobinaemia occurs in many cases without oliguria (Creevy, 1948; Biorn and Greene, 1949), and its degree bears no relationship to the severity of the renal failure. Whilst a number of isotonic solutions have been employed as irrigating fluids in prostatic resection (Ebert, 1949; Nesbit and Glickman, 1948; Garske, Phares, and Sweetser, 1949) we have used isotonic glucose solution, which does not cause haemoglobinaemia.

We have found that the oliguric syndrome has not been eliminated by the use of solutions which avoid haemoglobinaemia, as has been asserted (Chapman and Sutherland, 1952). Further, in some of our cases, transfusion, sulphonamide therapy, and clinical shock played no part, though these factors have been present in many reported cases of lower nephron nephrosis, and in most of those associated with transurethral prostatectomy.

Our six cases all developed oliguria or anuria. Water irrigation was employed in Cases 1 and 2; 5% glucose in Cases 3 to 6 (though in Case 3, for technical reasons, water was substituted for glucose during the operation). Their clinical and pathological findings are briefly described.

Case Reports

Case 1.—J.N., aged 70 years, had benign prostatic obstruction, for which prostatic resection was performed on November 10, 1949, when 25 g. of prostatic tissue were resected. He had oliguria the following day, and was completely anuric for 73 hours until his death in uraemic coma on November 16.

Necropsy was performed 36 hours after death.

In addition to the renal lesion, there was extensive bilateral bronchopneumonia, confluent in both lower lobes. The kidneys together weighed 350 g. They showed considerable scarring, and on bisection appeared to consist almost entirely of pale, swollen, cortical tissue, which bulged over the capsule.

Histology.—The glomeruli showed no abnormality. The cells of the first convoluted tubules were flattened and the lumen of the tubules wide. No necrosis was present. A few small areas of complete tubular degeneration were found in the outer medulla, the cells of the

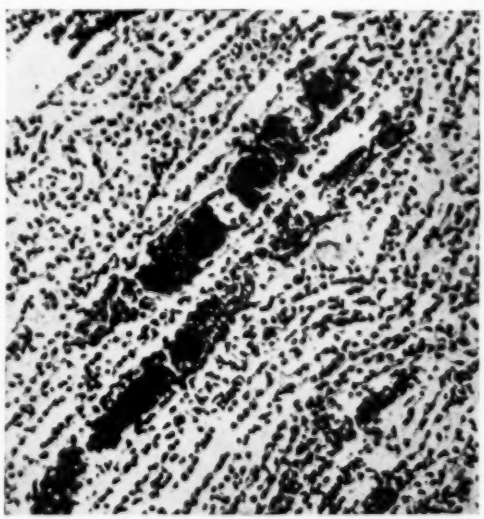


FIG. 1.—Granular casts in collecting tubules in Case 1. Haematoxylin and eosin, × 100.

tubules having disintegrated into a uniform granular material. Some polymorphic infiltration was seen in the interstitial tissue around these areas of necrosis.

Many of the second convoluted tubules contained copper-coloured, granular casts. Some of the tubules containing these casts showed no obvious abnormality of their lining cells. Others had grossly abnormal lining cells which were swollen and darkly staining. An occasional cast was scantily infiltrated with polymorphs.

There were very many pigmented, granular casts (copper-coloured in haematoxylin-and-eosin-stained sections) in the collecting tubules (Fig. 1); the interstitial tissue around some of these tubules was scantily infiltrated with polymorphs and lymphocytes, but the cells of the collecting tubules appeared normal. A few small foci of cellular infiltration were present around tubules not containing casts, and lymphocytic foci were present in the walls of some interlobular veins.

These findings are characteristic of lower nephron nephrosis. In the description of the subsequent cases only points of difference from the above are described fully.

Case 2.—A.G.M., aged 61 years, had benign prostatic obstruction, for which transurethral resection was performed on January 4, 1950, when 15 g. of prostatic tissue were resected.

Oliguria was present, with a progressive rise of blood urea to 192 mg.% on January 14 when diuresis occurred, and the blood urea had reverted to normal by January 19. The patient developed bronchopneumonia and died on January 20.

Necropsy was performed 48 hours after death.

In this man death was largely due to a severe upper respiratory infection and early bilateral bronchopneumonia.

The left kidney appeared normal; the right was pale and swollen, but showed no other gross abnormality.

Histology.—The right kidney showed severe early pyelonephritis. The left kidney, though macroscopically normal, showed the following histological lesions. Some collecting tubules contained brown, granular pigment, particularly prominent in the large tubules near the apex of the pyramid. One cast was infiltrated with polymorphs. In the outermost medullary zone were numerous, circumscribed, small collections of chronic inflammatory cells, in and around small segments of tubules. These cells were mostly plasma cells and lymphocytes with an occasional eosinophil and polymorph, and a small number of fibroblasts. Fragments of cast material were present in a few tubules in this area. Similar cellular collections were present in the immediate vicinity of a few glomeruli, particularly in the inner cortical zone. One large recent thrombus was present in a thin-walled vein in this zone.

Unfortunately the histology of the first convoluted tubules was obscured by severe post-mortem degeneration.

These lesions apparently represent the healing phase. This man had been oliguric for 10 days, but recovered clinically from the uraemia which followed the operation.

Case 3.—J.H., aged 87 years, had a carcinomatous prostatic obstruction with retention. He was grossly anaemic and this was treated initially. Transurethral resection was performed on December 15, 1950, 5 g. of prostatic tissue being resected. He immediately became anuric, and died on December 21 in uraemic coma.

Necropsy was performed eight hours after death.

Outside the renal tract the only abnormalities were the presence of numerous small pigment stones in the gall-bladder and a severe zonal fatty change in the liver.

The kidneys together weighed 180 g., and showed the gross appearances of severe atherosclerosis.

Histology.—The first convoluted tubules were dilated and contained a little albuminous material, and the cells showed considerable vacuolation of the cytoplasm, but the nuclei were well preserved. In a few collecting tubules and in a small number of second convoluted tubules in the cortex there was very scanty granular red pigment. Some extratubular pigment closely resembling the more usual intratubular material lay in the interstitial tissues of the medulla. Small foci of necrotic second convoluted tubules were present in the cortex, and necrotic loops of Henle in the outer medulla.

Case 4.—J.M., aged 71 years, had benign prostatic obstruction with acute retention. Transurethral resection of 20 g. of tissue was performed on January 26, 1951. Thereafter the patient was oliguric for 48 hours, and then completely anuric until his death in uraemic coma on February 6.

Necropsy was performed one and a half hours after death.

Apart from the kidneys, the only abnormality found was an enlarged heart with an old posterior infarction. The kidneys together weighed 440 g., and showed marked foetal lobulation. The cortex was thickened, averaging 10–11 mm. and in some places 15 mm. across.

The medulla was pale except for a 1 mm. thick, congested line at the cortico-medullary margin.

Histology.—The first convoluted tubules were well preserved but were very dilated. Some pink, rather solid-looking debris was present in the second convoluted tubules and loops of Henle in the cortex. In the cortex small vascular and cellular scars surrounded the distorted and regenerating second convoluted tubules.

The outer area of the medulla showed the most marked abnormality. Here, corresponding to the anatomical position of the loops of Henle, numerous tubules contained giant and distorted, deeply staining cells, the interstitial tissue being oedematous and scantily infiltrated with chronic inflammatory cells. The majority of the tubules in this zone showed marked evidence of cellular regeneration (Figs. 2 and 3).

Case 5.—C.B., aged 78 years, had a carcinomatous prostatic obstruction. On June 15, 1951, 5 g. of prostatic tissue were resected, after which anuria developed and the patient died in uraemic coma on June 20.

Necropsy was performed six hours after death, but permission for a general necropsy was refused.

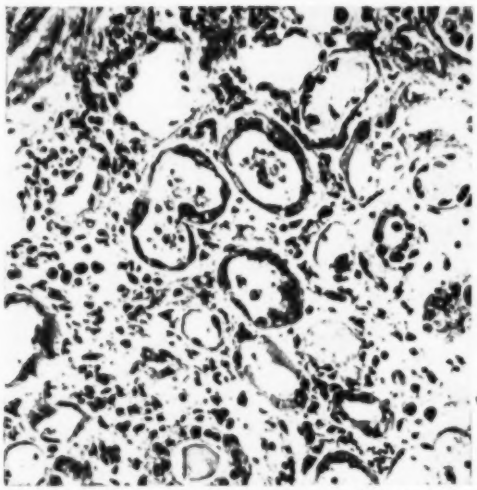


FIG. 2.—Cellular regeneration in the tubules in Case 4. Haematoxylin and eosin, × 160.

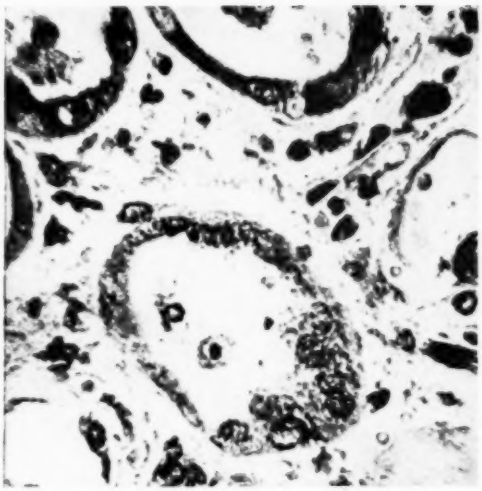


FIG. 3.—Cellular regeneration in the tubules in Case 4. Haematoxylin and eosin, × 560.

There were numerous small areas of acute infection in one kidney and a few similar areas in the other.

There were many pigmented casts in the second convoluted tubules, collecting tubules, and in some loops of Henle.

Only one small focus of necrotic tubules in the juxtamedullary zone was seen. There were several cortical foci of necrotic and regenerating tubules near the glomeruli with and without cast material. Two blue-staining casts (in haematoxylin-and-eosin-stained preparations) surrounded by giant cells were seen.

One unusual feature was the presence of intratubular red cells, sometimes associated with cast material, in the loops of Henle, in two glomerular spaces, and in immediately adjacent proximal convoluted tubules. The number of red cells in some of the lower tubules was suggestive of direct bleeding into these tubules rather than of the passage of blood from the glomerular space. Even a small quantity of blood in the glomerular space makes one reluctant to accept tubulo-venous anastomosis as the origin of the intratubular blood, as we did not find tubulo-venous anastomosis in any of the kidneys examined. The intratubular bleeding in this case was probably not directly associated with the lower nephron nephrosis.

Case 6.—J.C., aged 65 years, had a benign prostatic obstruction.

On August 1, 1951, 19 g. of prostate were resected, and a transfusion of two pints of blood given. On August 4 oliguria began, and on August 8 he was anuric, and died in coma on August 9.

Necropsy was performed 22 hours after death.

Macroscopically the liver showed gross fatty change. There was a severe, haemorrhagic inflammation of the pelvic peritoneal tissues as high as the lower abdominal wall anteriorly and the mid-lumbar vertebrae posterorily. No actual pus was found.

Kidney tissue was obtained within five hours of death. The kidneys together weighed 290 g., their only visible abnormality being pallor and some swelling of the cortex, which was 10 mm. thick in some places.

Histological examination confirmed the severe fatty infiltration of the liver.

There was some swelling of the cells of the proximal convoluted tubules, congestion of the glomeruli, and

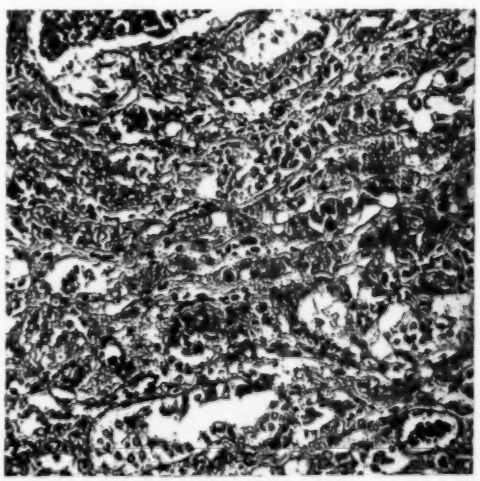


FIG. 4.—Necrotic tubules in Case 6. Haematoxylin and eosin, × 100.

small foci of necrotic second convoluted tubules in the cortex.

Many entirely necrotic tubules were present in the outer medulla, corresponding to the anatomical position of the loops of Henle. In many tubules no cells were seen, only granular debris within an ill-defined basement membrane remaining (Fig. 4). In a few tubules some large regenerating cells, showing mitosis, were present. Some necrotic areas, and the walls of a few large veins, showed scanty polymorphic infiltration.

Pigmented casts were not found in these kidneys.

Discussion

Similar lesions to those we have described have been seen in many clinical conditions. Bywaters and Dible (1942) gave prominence to the lesion in describing the anuria which may follow crushing injuries. The largest group of cases described, battle casualties, is that of Lucké (1946), and he and Mallory (1947) emphasized the variation in the amount of tubular damage and cast formation. The most marked change is in the distal tubules, notably the ascending limb of Henle's loop and the second convoluted tubule.

Changes in the proximal convoluted tubules have been described by many authors: for example, distension (Baker and Dodds, 1925), tubular degeneration (Hellwig and Reed, 1942; Lederer and Rosenblatt, 1942), and pigment cast formation (McLaughlin, Holyoke, and Bowler, 1947). Bell (1950) believes that hydropic degeneration of the proximal tubules is the essential lesion. Changes in these tubules were present in three of our cases, but in no case did we see actual necrosis, and we agree with Ayer and Gauld (1942) that it is "impossible to attach any great significance to any of these changes."

As stated above, there may be great variation in the degree of damage to the lower nephron, and although it sometimes appears insufficient to account for the loss of renal function, in some instances the damage certainly is sufficient. In Case 6 the necrosis involved a band of tubules in the outer medulla so severely that all the ascending limbs of the loops of Henle were destroyed. In Case 4 a similar extensive zone of grossly abnormal regenerating tubules was seen. In the other cases the areas of necrosis in the loops of Henle and second convoluted tubule were patchy and focal in distribution. It seems, therefore, that the name "lower nephron nephrosis" adequately emphasizes the anatomical location of a lesion which can nearly always be demonstrated.

Casts of a hyaline and granular type are often found in this condition, and their typical distribution is in the distal tubules. They provide an easily recognizable feature, and when present should at once direct the attention of the histologist to the condition of the cells in the lower tubules, particularly in the outer medulla. If the clinical history suggests the possibility of lower nephron damage, search for it is automatic. In the absence of suggestive history, the necrotic lesions, particularly if they are scanty or focal, and if pigmented casts are absent or few, may well be overlooked (Creevy, 1948). The longer post-mortem examination is delayed, the more likely is the lesion to be overlooked. Advanced post-mortem change may make it impossible to determine whether or not ante-mortem necrosis was present, and in the absence of typical casts it may not be possible to reach a diagnosis. Further, organs which show severe post-mortem change tend to receive less careful attention from pathologists. We feel, therefore, that this lesion may be more common than is suggested by the literature or standard text-books.

In the British literature no account has been published of lower nephron nephrosis complicating prostatectomy, though Wardill (1946) was probably the first to observe its occurrence. We know of no case in which the lesion was found after an open prostatectomy, and its occurrence after transurethral resection may be due to some factor in the operative technique in that procedure.

In prostatic operations, blood loss (and possible transfusion), anaesthesia, and surgical shock are factors common to all. Further, the lesion occurs in the absence of recognizable surgical shock and without transfusion. When oliguria follows transfusion, the possibility that the transfusion was incompatible should always be considered. In only one case in this series was a transfusion given, and there was nothing to suggest that this was incompatible. There was no prolonged fall of blood pressure in any of the cases.

An important difference between transurethral resection and all the other forms of prostatectomy is that in the former the bladder is filled and emptied continuously throughout the operation. The irrigating fluid, be it water, 5% glucose, glycine solution, or mannitol, is absorbed from the bladder into the blood stream through the prostatic veins (Creevy, 1948; Bunge and Barer, 1948). There is convincing evidence that prostatic fluid enters the blood stream during operation. The piecemeal cutting of the prostate releases secretion, and some of this, along with the irrigating fluid, enters the circulation through the open wound at the operation site.

Post-operative Rise in the Serum Acid Phosphatase.—A high concentration of phosphatase is present in the effluent from the bladder during the

operation. In one case, after the resection of only 2 g. of tissue, the effluent contained 1,600 units of formalin-stable acid phosphatase per 100 ml. Absorption into the blood stream also occurs, as a small but definite rise in the serum acid phosphatase is found in almost every case following resection, and often the rise is dramatic. The greatest rises follow large resections, but the rise is not always proportional to the amount of tissue removed. This is not surprising, since the rise must depend on two factors, apart from the amount of tissue resected. These are the amount of phosphatase actually present in the tissue removed, and the amount of irrigating fluid which enters the blood stream. The former varies considerably, since the amount of acinar and fibro-muscular tissue in the hypertrophied prostate varies greatly, and the latter depends on the length of the operation, the fluid pressure in the bladder, and the number and size of the opened vascular channels.

TABLE

SERUM ACID PHOSPHATASE LEVELS* IMMEDIATELY BEFORE AND AFTER OPERATION

Prostatic Tissue Removed (g.)	Serum Acid Phosphatase (units per 100 ml.)	
	Before Operation	After Operation
Transurethral prostatectomy (resection)		
4	0·75	3·0
4	2·25	3·4
4	2·0	19·8
6	2·7	2·7
6	2·4	7·9
14	2·3	30·0
15	1·5	6·4
17	1·0	34·0
18	1·1	19·2
20	2·7	41·8
23	2·1	53·5
25	1·4	12·5
45	3·5	86·0
49	1·2	39·2
Open prostatectomy (enucleation)		
40	2·4	2·7
43	0·25	0·8
52	1·0	5·1
90	2·8	4·2

*Formaldehyde stable—Abul-Fadl and King, 1948.

The accompanying table shows that the rise in phosphatase level is small with small resections, but when over 10 g. tissue are removed the rise is usually great. That this rise is due to the method of removal rather than mere manipulation of the prostate is shown by comparing these results with the phosphatase levels following prostatic enucleation. In the latter, despite the large glands removed, the rise was extremely small, being of the same order as, or less than, that resulting from the removal of one-tenth to one-twentieth of the tissue by resection.

The fall to normal takes 24–36 hours, being longer when the post-operative rise is great. In the largest rise recorded (to 86 units) the phosphatase was 17 units seven hours after operation, and 2.5 units 31 hours after. When the post-operative level was 39.2 units, the level four hours and 24 hours post-operatively was 9.3 and 4.5 units respectively. When the post-operative level was 12.8 units, it had dropped to 0.75 units after 24 hours.

Clearly routine acid phosphatase levels should not be measured for 24 hours after enucleation and not for two days after the operation of resection. We have not ascertained whether prostatic massage or digital examination of the prostate per rectum influences the serum level.

We have also been unable to demonstrate any relationship between the rise in serum acid phosphatase, which indicates absorption of prostatic secretion into the blood stream, and the occurrence of tubular necrosis, and work is proceeding to investigate this further.

Summary

Six cases of lower nephron nephrosis following transurethral resection of the prostate are described; isotonic solutions as irrigating fluids have not eliminated this risk.

Lower nephron nephrosis does not occur after open prostatectomy.

Absorption of prostatic secretion from the bladder during resection is shown by the dramatic rise of serum acid phosphatase; during enucleation of the prostate a very small rise of phosphatase is observed. We have no evidence that the renal lesion is related to the absorption of secretion.

We would like to thank Dr. R. P. Smith and Dr. J. Hart-Mercer for their help and advice, and Mr. A. Hall for the photomicrographs.

References

Abul-Fadl, M. A. M., and King, E. J. (1948). *J. Path. Bact.*, **60**, 149.
Ayer, G. D., and Gauld, A. G. (1942). *Arch. Path., Chicago*, **33**, 513.
Baker, S. L., and Dodds, E. C. (1925). *Brit. J. exp. Path*, **6**, 247.
Bell, E. T. (1950). *Renal Diseases*, 2nd ed. London.
Biorn, C. L., and Greene, L. F. (1949). *Surg. Gynec. Obstet.*, **88**, 389.
Bratton, A. B. (1941). *Lancet*, **1**, 345.
Bunge, R. G., and Barer, A. P. (1948). *J. Urol., Baltimore*, **60**, 122.
Bywaters, E. G. L., and Dible, J. H. (1942). *J. Path. Bact.*, **54**, 111.
Chapman, T. L., and Sutherland, J. W. (1952). *Brit. med. J.*, **1**, 72.
Creevy, C. D. (1947). *J. Urol., Baltimore*, **58**, 125.
—— (1948). Ibid., **59**, 1217.
Ebert, C. E. (1949). Ibid., **62**, 736.
Foy, H., Altmann, A., Barnes, H. D., and Kondi, A. (1943). *Trans. roy. Soc. trop. Med. Hyg.*, **36**, 197.
Garske, G. L., Phares, O. C., and Sweetser, T. H. (1949). *J. Urol., Baltimore*, **62**, 322.
Goodpastor, W. E., Levenson, S. M., Tagnon, H. J., Lund, C. C., and Taylor, F. H. L. (1946). *Surg. Gynec. Obstet.*, **82**, 652.
Hellwig, C. A., and Reed, H. L. (1942). *J. Amer. med. Ass.*, **119**, 561.
Lederer, M., and Rosenblatt, P. (1942). Ibid., **119**, 8.
Lucké, B. (1946). *Milit. Surg.*, **99**, 371.
Mallory, T. B. (1947). *Amer. J. clin. Path.*, **17**, 427.
McLaughlin, W. L., Holyoke, J. B., and Bowler, J. P. (1947). *J. Urol., Baltimore*, **58**, 47.
Nesbit, R. M., and Glickman, S. I. (1948). Ibid., **59**, 1212.
Wardill, W. E. M. (1946). *Brit. J. Urol.*, **18**, 72.
Young, J. (1942). *Brit. med. J.*, **2**, 715.

J. clin. Path. (1952), 5, 239.

ANISOTROPIC NON-SILICIOUS CRYSTALLINE INCLUSIONS IN A DYSGERMINOMA OF TUBERCULOID APPEARANCE

BY

R. ERIC POTTS

From the Pathology Laboratory, Kilmarnock Infirmary, Ayrshire

(RECEIVED FOR PUBLICATION DECEMBER 17, 1951)

It is widely recognized that in lesions involving the ovary and Fallopian tube the histological appearances of dysgerminoma on the one hand, and talc granuloma on the other, may in some instances resemble those of tuberculosis. Conversely, the lesions of tuberculous salpingitis may also, in certain cases, closely mimic those of tubal carcinoma or tubal talc granuloma. In these circumstances crystals in lesions of the Fallopian tube assume diagnostic significance. Roberts (1947) called attention to the lesion of tubal talc granuloma and demonstrated the silicious nature of the doubly refractile crystals therein, and Potts (1951) recorded the occurrence of non-silicious anisotropic crystals in tuberculous salpingitis. It is the purpose of the present communication to record the finding of birefringent, intracellular, non-silicious crystals in a dysgerminoma of tuberculoid appearance involving the Fallopian tube and to discuss the diagnostic implications of this observation.

Case Report

M.S., an unmarried woman of 23 years, was admitted to hospital on August 18, 1950, complaining of right-sided abdominal pain of several weeks' duration. She gave no history of previous surgical intervention. A hard mass, probably ovarian, was palpable in the right lower abdomen and at operation the mass was confirmed as a tumour of the right ovary. The right Fallopian tube was adherent to the tumour which apparently involved it, and there was a further mass behind the mesentery in the retroperitoneal tissues. The left ovary and tube appeared healthy.

Pathology

The specimen was an ovoid mass of 9 cm. diameter replacing the right ovary and continuous with the related Fallopian tube of which 5 cm. was present. The ovarian tumour had a smooth surface and was bounded by a tough capsule, and in slices it was firm and grey with numerous small irregular yellow areas. The distal half of the portion of Fallopian tube was enlarged up to 2 cm. maximum diameter and on section showed a small eccentric lumen and much thickening of the wall and adjacent broad ligament of a similar appearance to the ovarian tumour.

Histologically, the ovarian tumour and its tubal extension presented two quite dissimilar pictures. In the lesser part of the considerable tissue sampled the neoplasm presented the typical appearance of a dysgerminoma (Fig. 1). The neoplastic cells were large, round, and uniform, and each had a large

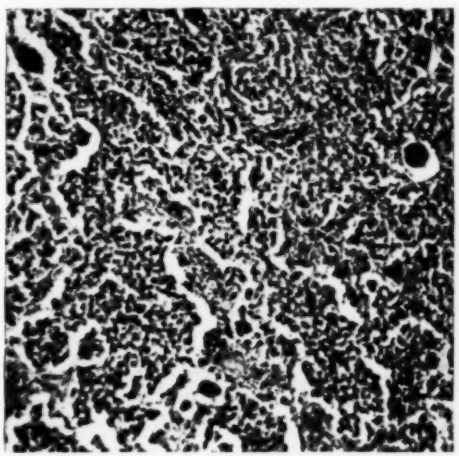


FIG. 1.—Dysgerminoma of typical pattern. (Haematoxylin and eosin, × 35.)

vesicular nucleus and abundant, clear, and translucent, or even slightly basophilic, cytoplasm. The nucleus was rich in chromatin in a coarsely granular distribution and featured one, or occasionally more than one, prominent nucleolus. At a magnification of 480 diameters, mitotic figures averaged 2.5 per field. The cells were supported by a delicate fibrous stroma and arranged in characteristic

FIG. 2.—Dysgerminoma of follicular pattern in which many of the follicles contain one or more darkly staining giant cells. (Haematoxylin and eosin, × 35.)

alveolar fashion, while the bands of fibrous tissue presented not only the usual lymphocytic infiltration but also numerous plasma cells.

In the major part of the material, however, there was at low magnification a superficial resemblance to non-caseous tubercle in the stage of early fibrous healing (Fig. 2). Follicles or islets of palely staining cells of irregular outline were circumscribed by abundant fibrous tissue infiltrated, in lesser or greater degree, by lymphocytes. Many of the follicles contained one, or more than one, multinucleate giant cell of the order of 100 microns and occasionally much larger. At higher magnification the epithelioid cells forming these follicles were not of uniform type. Some appeared as poorly outlined, interwoven cells with palely eosinophilic or clear cytoplasm and a small vesicular nucleus similar to the epithelioid cells found in the "soft" tubercle; others were frankly neoplastic cells readily identifiable by their much larger and coarser nuclei and prominent nucleoli, while in many the cell nuclei showed varying degrees of karyolysis, so that it was not possible to classify them with certainty as neoplastic or reactive. Of the multinucleate giant cells which formed a prominent feature of many of the follicles, a few showed the peripheral nuclear arrangement of the Langhans type of cell, but the majority presented a widely scattered nuclear distribution. The multiple nuclei, each about one third of the size of the nucleus of the average tumour cell, were oval in shape and poor in chromatin, while the cytoplasm was deeply eosinophilic and frequently vacuolated. Other multinucleate cells, of smaller size and having fewer, larger, and more deeply stained nuclei, were clearly symplastic tumour giant cells, and again some of the multinucleate cells could not be classified. At the margin of the follicles the peripheral fibroblastic reaction rapidly gave place to the abundant collagenous tissue which separated the individual follicles. Occasionally small foci consisting of one or two foreign-body giant cells and a few macrophages, lymphocytes, and plasma cells were encountered in the stroma far removed from recognizable neoplastic cells (Fig. 3). Not only were lymphocytes present in the peripheral fibrous boundary zone of the follicles, but plasma cells were also numerous and in parts predominant, while a proportion of cells were

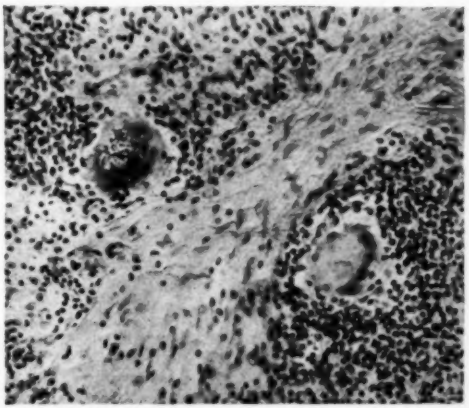


FIG. 3.—Two large multinucleate giant cells photographed by direct illumination and through crossed polaroid screens. The foreign body type of cell contains an aggregation of birefringent crystals. No inclusions are present in the Langhans cell. (Haematoxylin and eosin, × 170.)

identified as large mononuclears by their abundant cytoplasm and nuclei similar to those of the foreign-body giant cells. Crystalline inclusions were present in many, but by no means all, of the multinucleate giant cells (Fig. 3) and were also found occasionally in degenerate tumour cells or macrophages, but since the nuclear staining of these latter cells was commonly absent they could not be classified. The crystals appeared translucent or pale yellow, were inconspicuous by transmitted illumination, but were strongly birefringent. They were variable in size up to 50 microns, and the larger examples were considered as aggregations of smaller crystals since different parts of the same mass "blacked out" at different degrees of rotation of the slide between crossed polaroid screens. They were always readily soluble in dilute mineral acids within 30 minutes; indeed most dissolved within five minutes. While usually occurring alone, a few of the crystals were intimately associated with basophilic laminated bodies (Fig. 4) of the order of 10 to 50 microns or even larger, while these latter also occurred sometimes independently within giant cells or extracellularly in relation to such cells. They appeared morphologically similar to the bodies encountered from time to time in the lesions of tuberculosis and sarcoidosis, and, like them, gave positive results with stains for iron.

Sections taken from the proximal portion of the specimen of Fallopian tube were without evidence of tumour invasion or of chronic inflammatory changes, and numerous sections from the distal portion, in which a tuberculoid appearance was apparent, showed no acid-fast organisms. Glycogen was easily demonstrable by the periodic acid-Schiff technique (with saliva control) in both the tuberculoid and the typical areas of the tumour. Frozen sections stained by Sudan black showed diffuse dark grey staining of the cytoplasm of the majority of the multinucleate giant cells, but fatty material in the tumour cells was not demonstrated.

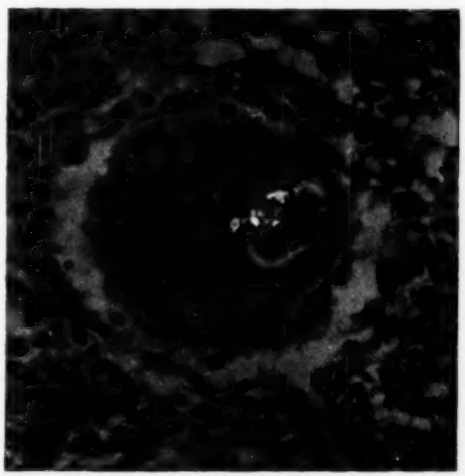


FIG. 4.—A large multinucleate giant cell of foreign-body type photographed through partially crossed polaroid screens to show the intimate association of birefringent crystals with an isotropic concentric laminated body. (Haematoxylin and eosin, × 450.)

R

Discussion

The Tuberculoid Type of Lesion.—In a paper devoted to the subject of dysgerminoma and tuberculosis Schiller (1934) concluded that the tubercle-like follicular lesions encountered occasionally in such tumours were not of tuberculous aetiology. In a review of the literature on the subject he classified as tuberculoid dysgerminoma five cases previously reported by Kermauner as coexisting granulosa cell tumour and tuberculosis, and several cases previously reported by various other authors as coexisting dysgerminoma and tuberculosis. In his Case 7, which bore a very close resemblance to tuberculosis, not only were suitably stained sections negative for tubercle bacilli but animal inoculation of the tissue and the tuberculin reaction of the patient were also negative. He, therefore, suggested that the tissue reaction was produced by fatty substances derived from the degeneration of the tumour cells. In contrast to this view that the epithelioid cells and multinucleate giant cells were a product of the stromal reaction, Novak and Gray (1938) regarded them as degenerate neoplastic cells, the giant cells in their opinion being symplastic in nature. Föderl (1938) found pseudo-tubercles in tumour thrombi lying within the lumina of small vessels, and, as no stromal elements were found, he assumed an origin either from tumour cells or endothelial cells, while he also pointed out that dysgerminoma cells were themselves mesenchymal derivatives. In the five cases of dysgerminoma reported by Sailer (1940), two presented a few pseudotubercles, but the feature was not prominent and he expressed no opinion regarding their origin. More recently, however, Heller (1943) in a study of two cases of tuberculoid dysgerminoma reached a conclusion similar to that of Schiller, while Potter (1946) in reporting a single case referred to the frequent presence of giant cells resembling Langhans cells, but gave no views as to their development. In the case presented here, the resemblance to tuberculosis was comparatively superficial as detailed study of the follicles showed significant differences from the follicular lesions of tuberculosis.

Of these the most striking was the vascularity; small capillary vessels were frequent within the central mass of pale epithelioid cells. A further differential point was that lymphocytes were not confined to the periphery of the follicles but were seen at focal areas within the epithelioid cell core or even diffusely scattered throughout. The cytological classification of the epithelioid cells was, however, a matter of greater difficulty, and the two widely differing views regarding the origin of these cells and the multinucleate giant cells were readily appreciated. The tuberculoid appearance was confined to those areas where degenerative changes in the tumour cells were prominent, and in the process of degeneration the cytoplasm of such cells becomes eosinophilic. Furthermore, the nuclei of the neoplastic cells showed varying degrees of karyolysis and, while generally large, exhibited the range in size usual to nuclei in poorly differentiated neoplastic tissue. There were, too, some small multinucleate protoplasmic masses clearly formed by fusion of three or four degenerate tumour cells. On the other hand, the frequent occurrence of deeply eosinophilic giant cells of the order of 100 microns containing upwards of 10, and occasionally more than 30 small uniform nuclei, left little doubt that these were true foreign-body giant cells and as such were to be regarded as derived from histiocytes. For the reasons given above it was not possible to identify with certainty macrophages within the islets, but at the periphery of the follicles such cells could be recognized in the fibroblastic tissue along with lymphocytes and plasma cells. Plasma cells were seen not only in the fibrous stroma in the tuberculoid parts of the tumour, but also in the supporting trabeculae of the more histologically typical areas of the neoplasm. These cells were also a feature of the lesion in the cases reported by Heller (1943) and he regarded them as supporting the hypothesis of an inflammatory or stromal origin of the epithelioid cells of the follicles. This argument appears to ignore the frequent occurrence of plasma cells in the supporting stroma of diverse neoplasms. Heller also commented on the presence of eosinophils, but these cells were not identified in the present material although occasional plasma cells presented eosinophilic Russell bodies. In my own view the epithelioid cells of the follicles are to be interpreted in part as degenerating tumour cells and in part as macrophages engaged in the scavenging thereof, while the multinucleate giant cells are similarly of neoplastic and reactive origin, that is, of both symplastic and foreign body type. A tuberculoid appearance in neoplasms is not confined to dysgerminoma: such a histological picture has been reported in neurocytoma (Hartz and van der Sar, 1945) and in granulosa cell carcinoma (Schattenberg and Harris, 1946). In the former case the authors concluded that "it may be safely assumed that the epithelioid cells were of mesenchymal origin," while in the latter case the theory of Schiller was regarded as the probable explanation, although no frozen sections were made to confirm the presence of fatty material. Schiller (1934) originally postulated that the tuberculoid tissue reaction in dysgerminoma was produced by fatty substances resulting from degeneration of the tumour cells, and in a later communication he (Schiller, 1936) noted that "a fat stain will reveal abundant lipoid," but he did not specify either the stain used or the site of the lipoid. Greenblatt and Pund (1938), however, were only able to find occasional macrophages and giant cells containing sudanophilic material. This latter finding was my own experience in material from this case, although it must be noted that the tissue was stored in formalin for several months before frozen sections were cut. While neutral fats are unaffected by such treatment, phospholipines form fine emulsions in water and gradually disappear from tissues stored in formalin. On the other hand, it is worthy of note that glycogen was readily demonstrable, though not abundant, in the tumour cells—yet another contradiction of the still frequently repeated fallacy that alcoholic fixation is necessary for the demonstration of this substance. Greenblatt, Greenhill, and Brown (1939) investigated the lipid content of various ovarian neoplasms, and stated that fatty material might be found in such tumours, due either to degenerative changes or to tissue metabolism (hormone storage); in dysgerminoma the fat was considered as degenerative in origin. The present case provides no evidence for or against the presence of lipid material in the degenerate areas of the tumour, since any such lipid might be expected to be phospholipine rather than neutral fat and as such would be removed by storage in an aqueous fixative.

Crystals.—So far as I am aware, the presence of birefringent crystals in a dysgerminoma has not hitherto been reported. The occurrence of such crystals in a tumour of tuberculoid histological appearance involving the Fallopian tube is noteworthy in that anisotropic crystalline inclusions are of some diagnostic significance in lesions involving a Fallopian tube and ovary. Roberts (1947) in a paper devoted to tubal silicious granuloma reported five such cases although only one was proved by micro-incineration. At the same time he commented on the similarity of the histological picture to that of tuberculous salpingitis and referred to the possibility of reaching this erroneous diagnosis in such lesions. On the other hand, Potts (1951)

called attention to the presence of acid-soluble, birefringent crystals in the giant cells of tuberculous salpingitis and remarked upon the converse possibility of wrongly labelling such lesions as talc granuloma. Diagnostic errors have in the past arisen in the interpretation of the tuberculoid type of dysgerminoma. While this pitfall is now widely recognized, there remains the possibility of misinterpreting a lesion showing anisotropic crystals within the giant cells for coexisting dysgerminoma and silicious granuloma. Illusory support for such a diagnosis might well be provided by the ubiquitous appendicectomy scar.

In the present case all the doubly refractile crystals were readily soluble in dilute mineral acids, and sections presenting such crystalline inclusions were, after 30 minutes' immersion in N/1 hydrochloric acid, consistently free from anisotropic material. It is not within the scope of this communication to discuss the chemical nature of these crystals. This will be the subject of a separate paper. It may, however, be said that the crystalline inclusions and those which are to be found in many cases of tuberculous salpingitis have so many properties in common, both chemical and physical, as to leave no doubt that they are identical.

Summary

A case is presented of an ovarian dysgerminoma involving the related Fallopian tube in which the histological appearance of the greater part of the tumour was tuberculoid.

The origin of the epithelioid cells and multinucleate giant cells is considered and the relevant literature is briefly reviewed.

Attention is drawn to the presence of non-silicious anisotropic crystals within some of the multinucleate giant cells and the diagnostic implications of this finding are discussed.

I am indebted to Dr. W. B. Davis for helpful suggestions in the preparation of this paper and to Mr. H. Gray for the photomicrographs.

REFERENCES

Föderl, V. (1938). *Arch. Gynäk.*, **165**, 392.
Greenblatt, R. B., Greenhill, J. P., and Brown, W. R. (1939). *Amer. J. Obstet. Gynec.*, **37**, 929.
—— and Pund, E. R. (1938). Ibid., **35**, 667.
Hartz, P. H., and van der Sar, A. (1945). *Amer. J. clin. Path.*, **15**, 473.
Heller, E. L. (1943). *Arch. Path., Chicago*, **35**, 674.
Novak, E., and Gray, L. A. (1938). *Amer. J. Obstet. Gynec.*, **35**, 925.
Potter, E. B. (1946). *Amer. J. Path.*, **22**, 551.
Potts, R. E. (1951). *Journal of Clinical Pathology*, **4**, 333.
Roberts, G. B. S. (1947). *Brit. J. Surg.*, **34**, 417.
Sailer, S. (1940). *Amer. J. Cancer*, **38**, 473.
Schattenberg, H. J., and Harris, W. H. (1946). *Amer. J. Path.*, **22**, 539.
Schiller, W. (1934). *Arch. Gynäk.*, **156**, 513.
—— (1936). *J. Obstet. Gynaec. Brit. Emp.*, **43**, 1135.

J. clin. Path. (1952), 5, 244.

THE HISTOLOGICAL CHANGES IN HYDANTOINATE GINGIVITIS

BY

L. CROME

From the Department of Neuropathology, the Fountain Hospital, London

(RECEIVED FOR PUBLICATION FEBRUARY 23, 1952)

The histological features of the gingival hypertrophy caused by the prolonged administration of hydantoinate drugs have been described by Glickman and Lewitus (1941), Ziskin, Stowe, and Zegarelli (1941), Zegarelli and Ziskin (1943), and by Esterberg and White (1945). The interval between the beginning dosage of the drug and the appearance of the gingival hypertrophy has varied within wide limits. Some cases were seen by Esterberg and White four years after its beginning, but neither they nor the previous workers have specified the duration of the gingival condition at the time of the biopsy. Some of the histological findings have given rise to concern over the possibility of malignant change intervening in the hyperplastic epithelium; Esterberg and White reported the opinion of certain pathologists that "malignancy could not be dismissed entirely." It is, therefore, of interest to record the following case in which histological examination has followed what might well be a longer period of continued epanutin administration than in those cases previously reported.

Case Report

The patient was admitted to the Fountain Hospital at the age of 3 years and 8 months with microcephaly, epilepsy, right-sided hemiplegia, and idiocy. She was given epanutin, gr. ¾ daily, for two years before her death, and hypertrophic gingivitis was noticed a year after treatment was begun, although it may have been present before. The patient died at the age of 12 in status epilepticus.

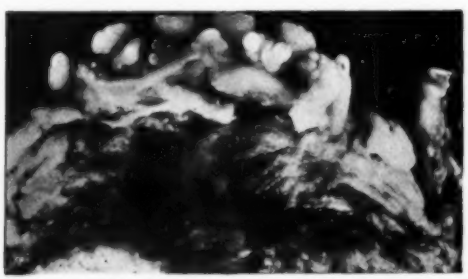


FIG. 1.—Papillary hypertrophy of mandibular gingival mucosa.

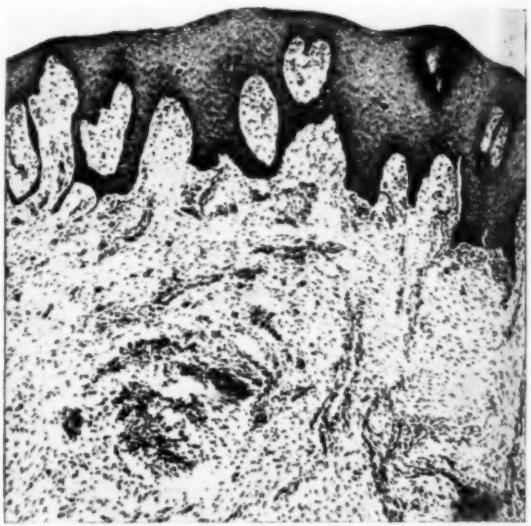


FIG. 2.—Acanthosis and granulomatous lesions in the corium. Haematoxylin and eosin, × 30.

Pathology

The examination of the gums revealed extensive gingival hypertrophy (Fig. 1). The principal microscopical changes consisted of acanthosis and chronic inflammatory infiltration of the corium (Fig. 2). In addition to the acanthosis, the Malpighian layer showed elongation of the rete pegs and proliferation of the basal layer. Mitotic figures were absent. A few epithelial pearls were present in the elongated rete pegs. The dermal papillae were long, narrow, oedematous, and contained some polymorphonuclear leucocytes, lymphocytes, and plasma cells. The corium was thickened and consisted of interlacing bundles of young collagen

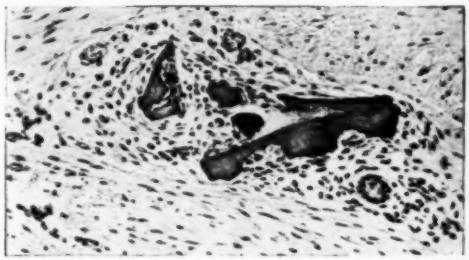


FIG. 3.—Partially calcified fragment. Haematoxylin and eosin, × 60.

and an excessive number of capillaries. In many areas there was perivascular accumulation of plasma cells and lymphocytes. Some of these plasma cells were multinucleated. A few partially calcified fragments could be seen in the deeper layers of the corium. These were formed by osteoid tissue with a central calcified core and possessed no Haversian canals (Fig. 3).

Discussion

All the structural features described above have been observed and reported previously, although not all of them in any single case. The inflammatory infiltration and the number of plasma cells appear to have been more conspicuous in the present case. The proliferation of the epithelial cells, and the formation of pearls and papillae were equally striking in this instance, but there was no evidence of any malignant change.

REFERENCES

Esterberg, H. L., and White, P. H. (1945). *J. Amer. dent. Ass.*, **32**, 16.
Glickman, I., and Lewitus, M. P. (1941). Ibid., **28**, 199.
Zegarelli, E. V., and Ziskin, D. E. (1943). *Amer. J. Orthodont.* (Sect. Oral Surg.). **29**, 152.
Ziskin, D. E., Stowe, L. R., and Zegarelli, E. V. (1941). Ibid., **27**, 350.

J. clin. Path. (1952), 5, 246.

THE VALUE OF RIB BIOPSY IN THE STUDY OF MARROW DISORDERS

BY

M. S. R. HUTT, P. SMITH, A. E. CLARK, AND J. L. PINNIGER

From St. Thomas's Hospital and Medical School, London

(RECEIVED FOR PUBLICATION FEBRUARY 7, 1952)

Specimens of marrow for histological examination may be obtained either by sectioning the particles present in a marrow aspirate or by surgical trephine of the sternum (Custer, 1933; Dameshek, Henstell, and Valentine, 1937; White, Baker, and Griffin, 1946). The former procedure is limited in many cases by the size of the particles withdrawn, while the latter, not infrequently, yields specimens consisting largely of bone and the marrow obtained may be distorted or too small for adequate histological study.

In view of these disadvantages it was decided to use rib biopsy as an alternative method for obtaining marrow for diagnostic purposes. The evaluation of normal variations and the selection and refinement of histological techniques were first undertaken on samples obtained at routine thoracotomies and at necropsy. Our findings are reported below.

Surgical Technique

The object of bone marrow biopsy is to produce a piece of bone with the medullary cavity undisturbed. This is most easily obtained, by one experienced in the technique, by resecting a small segment of rib subperiosteally. Although every patient was investigated before operation to exclude the probability of their bleeding excessively, this feature was not regarded as an absolute contraindication to the operation. In our series two cases showed haematomata at the site of operation, but these soon resolved with conservative treatment.

Of our 10 cases submitted to the procedure for diagnostic purposes, the first three were given local anaesthesia and the remainder general. The reason for the change-over was that it was more comfortable for the patient and was also a safeguard against accidentally opening the pleura. The operation is carried out in the theatre with full surgical asepsis and usually lasts about a quarter of an hour. A portion of rib $\frac{1}{2}$ to 1 in. long is removed from the midaxillary line according to the usual technique for subperiosteal resection. Haemostasis is simple and post-operative pain minimal.

Histological Technique

On receipt of the unfixed specimen smears are made from the marrow at one end of the rib either by direct impression or from particles removed with the point of a scalpel. These are dried in air, and fixed and stained with Leishman's stain as for ordinary marrow aspiration films.

The rib is then fixed for approximately 24 hours in 10% formalin neutralized with excess calcium carbonate, after which it is frozen with carbon dioxide snow. When frozen solid the rib is cut with a fine-tooth saw in the plane of its long axis and the greater diameter of its cut surface. Freezing before cutting ensures that the marrow is not torn away from the surrounding bone. This is particularly important if there is bony resorption secondary to marrow hyperplasia, when splintering is otherwise liable to occur.

The bisected rib is next decalcified with 5% nitric acid for four to eight hours. The usual procedure of neutralization with sodium sulphite is not carried out because we have found that the marrow cells show less shrinkage, and that the staining reactions, especially that with Leishman's stain, are much improved. The tissue is washed in running water for 24 hours, after which it is impregnated with and imbedded in paraffin wax in the usual way. Sections are cut at 4–5 μ and are attached to slides by using a gelatin technique, i.e., the sections are floated on to hot water to which has been added gelatin to make a 0.5% solution. They are withdrawn on to clean slides and then dried in the incubator in formalin vapour. This stage is necessary, particularly when haematoxylin and van Gieson and reticulin methods are to be used, as otherwise the sections will come off the slides.

The slides are stained with Ehrlich's acid alum haematoxylin and eosin, Weigert's iron haematoxylin, and Van Gieson's stain, with Leishman's stain, and for reticulin. The first two staining methods are standard ones and need not be discussed. The Leishman's stain is a modification developed by one of us (Clark, 1952) and has yielded results strikingly more satisfactory than any other method of which we have had experience.

The reticulin preparations have proved to be of great value in the study of the part played by connective tissue fibrils in haemopoietic disorders, and have given

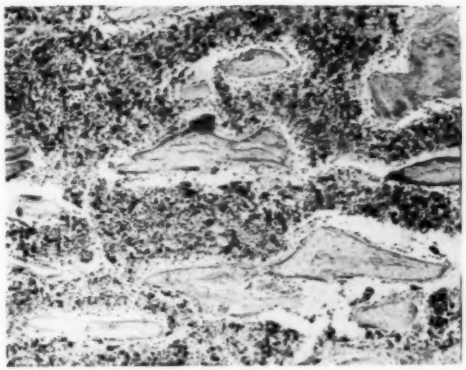


FIG. 1.—Normal rib from an infant showing numerous bone trabeculae and absence of fat spaces in marrow tissue. Haematoxylin and eosin, × 42.

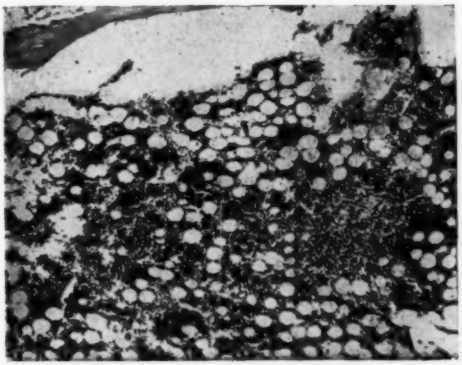


FIG. 2.—Rib from 20-year-old male showing haemopoietic tissue and fat spaces in normal proportions. Haematoxylin and eosin, × 42.

diagnostic information in cases in which the van Gieson preparation was disappointing.

Results

Normal Series.—A series of 50 ribs obtained from necropsies and during thoracotomies has been examined. All were treated according to the techniques described. It was found that suitable marrow specimens were obtained in all cases, though the earlier fixation that was possible in the surgical cases led to their having better cellular detail than those taken at necropsy.

In order to relate the changes in marrow content to age, specimens were examined from foetal life upwards to the age of 80. A rough estimate of marrow cellularity was obtained by counting the number of fat spaces in a random selection of six fields using the × 40 objective and × 6 eyepiece. In the two infants whose marrow was examined no such fat spaces were visible (Fig. 1); a few were present in a child of 4 and an average of 14 per field were seen in a boy of 12. There was not much variation from this figure after this age, even in the old-age group, and in these preparations haemopoietic tissue was always abundant and was similar to that in other sites (Fig. 2). This indicated that the rib was suitable for the study of bone marrow from patients of any age, confirming Reich's conclusions in the case of sternal marrow (Reich, Swirsky, and Smith, 1944).

Diagnostic Biopsy Cases.—The details of the 10 cases in which rib biopsy was undertaken for diagnostic purposes are given in the Table. In some of these the biopsy was undertaken because marrow punctures had yielded dry taps or dilute

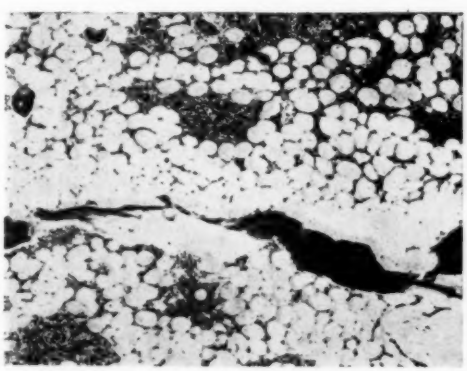


FIG. 3.—This section (Case 4) demonstrates the variation in cellularity that can occur. The islands are of normal haemopoietic tissue. Haematoxylin and eosin, × 42.

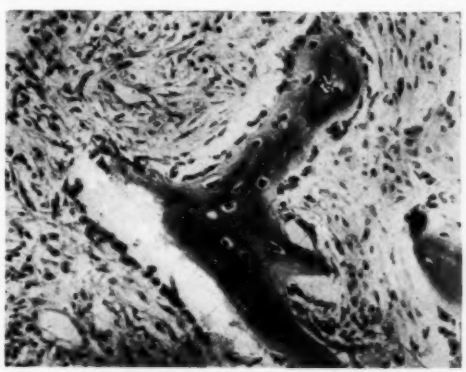


FIG. 4.—Marrow from Case 2 showing fibrosis with new bone formation. Haematoxylin and eosin, × 120.

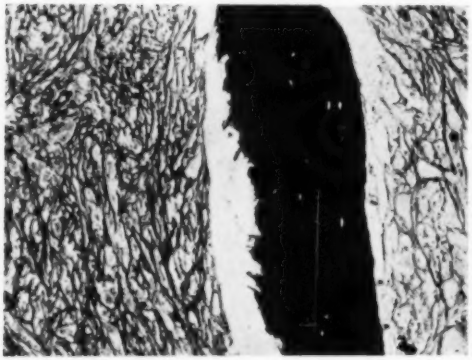


FIG. 5.—The same section as for Fig. 4. The fibrils are strikingly brought out by the reticulin stain. Van Gieson's stain was by contrast disappointing. Reticulin, × 120.

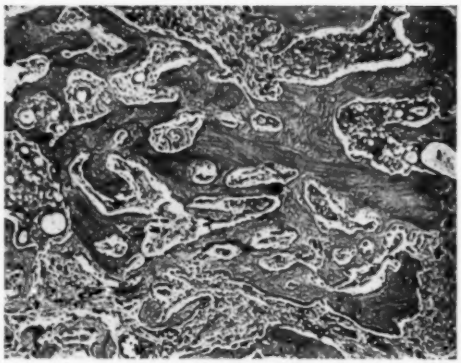


FIG. 6.—Secondary adenocarcinoma in bone marrow from Case 6. Active bone formation is well demonstrated. Haematoxylin and eosin, × 42.

specimens. On these occasions the biopsy specimen usually yielded diagnostic information, e.g., the presence of myelofibrosis in Case 1, of reticulum cell sarcoma in Case 3, and of secondary carcinoma in Case 6 (Fig. 6). Case 2 (Figs. 4 and 5) is an example of those leukaemic states which may be associated with an unsatisfactory marrow aspirate (Dacie and White, 1949). This case also illustrates the value of reticulin preparations in demonstrating the dense fibrillary nature of the connective tissue, a feature not apparent in the haematoxylin and van Gieson sections. Case 4 (Fig. 3) shows how false impressions of the marrow structure can at times be gained by relying on aspiration alone. Cases 2 and 10 (Figs. 7 and 8) give an indication of the value of marrow biopsy in the study of the evolution of interesting marrow disorders.

In Cases 5, 8, and 9 the biopsies were performed in order to obtain a clearer impression of the nature of the disorder and so come to a more confident diagnosis. In view of the fact that there were no serious post-operative complications we believe that rib biopsy is a useful additional tool in the comprehensive study of obscure haematological disorders, as it yields better histological preparations than any other method.

Conclusions and Summary

Our experience with rib biopsy leads us to the firm conclusion that it is in every way a more satisfactory procedure than sternal trephine in the study of the more obscure haematological disorders. While general anaesthesia is not imperative it is to be recommended. The operation, by one skilled in the technique, is easily performed, short, and

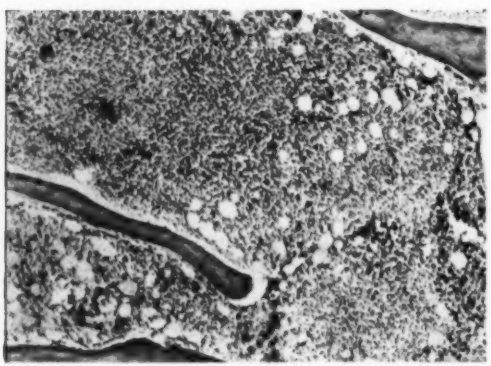


FIG. 7.—Marrow (Case 10) showing three rather ill-defined lymphoid follicles. Haematoxylin and eosin, × 42.

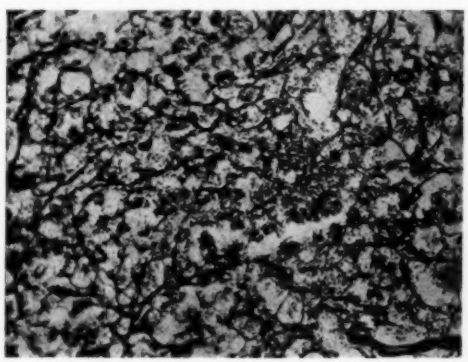


FIG. 8.—Marrow from Case 10 showing increase in number of reticulin fibrils. Reticulin, × 240.

TABLE

SUMMARIES OF RIB BIOPSY CASES

Case No.	Presenting Features	Marrow Puncture	Marrow Biopsy	Comments
1	Woman aged 74. Splenomegaly. Leuco-erythroblastic anaemia	Aspiration difficult. Marrow with normal differential count	Marrow fibrosis. Haemopoietic tissue present	
2	Man aged 53. Pancytopenia with atypical "blast" cells in peripheral blood	Marrow felt "hard." Scanty aspirate, having differential count similar to peripheral blood	Extensive marrow fibrosis with little haemopoietic tissue and scattered atypical primitive cells. (Figs. 4 and 5)	Studies indicated a complex state showing features of a primitive cell leukaemia and reticulum cell sarcoma
3	Man aged 29. Generalized lymphadenopathy. Neutropenia (Hb 100%)	Dilute marrow obtained with difficulty. Normal differential count	Normal marrow pattern destroyed. Increase in collagen and reticulin with reticulum cells and fibroblasts scattered through haemopoietic tissue	Gland biopsy revealed reticulum cell sarcoma
4	Man aged 60. Mass in left loin? spleen. No significant blood abnormalities	Marrow aspirate relatively acellular but otherwise normal	Section showed a fatty marrow in which foci of active haemopoiesis were present (Fig. 3)	Laparotomy revealed a renal tumour
5	Man aged 62. Refractory anaemia with leucopenia and splenomegaly	Marrow cellular. Showed normoblastic hyperplasia with an increase of immature forms and an occasional megaloblast	Active somewhat immature haemopoietic tissue with absence of fat spaces. No increase of fibrous tissue	Biopsy to assist in assessing advisability of splenectomy
6	Man aged 55. Malaise and loss of weight. Leuco-erythroblastic anaemia	Dry tap	Intense osteosclerosis due to secondary mucus-secreting carcinoma (Fig. 6)	
7	Woman aged 64. Long history of anaemia and leucopenia. Splenomegaly	Puncture twice performed yielded dilute marrow of normal differential count	Normal marrow	Rib biopsy was done at the same time as a liver biopsy. The latter showed multilobular cirrhosis
8	Man aged 63. Anaemia with slight leucocytosis. Occasional immature cell in peripheral blood. Diagnosed elsewhere as megakaryocytic myelosis	Marrow easily obtained. Differential count was of normal proportions. No megakaryocytic hyperplasia evident	Hyperplastic marrow with absence of fat spaces. Large numbers of myeloid cells and a great increase in the number of megakaryocytes	Marrow biopsy led to diagnosis of myeloid leukaemia with associated megakaryocytic hyperplasia
9	Woman aged 63. Splenomegaly with leucocytosis for three years. No anaemia. Occasional metamyelocyte in peripheral blood	Cellular marrow with myeloid hyperplasia	Marrow hyperplasia with absence of fat spaces. Myeloid leukaemia. No increase of fibrous tissue	Sternal puncture and rib biopsy were done at the same time under anaesthesia. The section showed a larger number of primitive cells than did the aspirate
10	Man aged 71. Splenomegaly. Anaemia and neutropenia. Occasional immature cell in peripheral blood	Marrow easily withdrawn, but dilute. The differential count was of normal proportions	The marrow showed an increased cellularity, the principal abnormality being a proliferation of reticulum cells. An unusual number of lymphoid follicles were present. There was a definite increase in the number of reticulin fibrils (Figs. 7 and 8).	As a result of marrow biopsy the case appeared to be one of early myelofibrosis

unlikely to be attended by any serious complications. The specimens obtained can be prepared without difficulty to give first-class marrow sections. In the staining of the latter, the orthodox haematoxylin and eosin and van Gieson stains and an improved Leishman technique are recommended. The particular value of silver methods for connective tissue fibrils is also stressed. The 10 cases in which biopsy was performed are tabulated and discussed. The use of the procedure as a tool in the comprehensive study of haematological disorders is to be recommended in addition to its undoubted value in diagnosis.

The expenses of this study have been defrayed in part by the Endowment Fund, St. Thomas's Hospital.

REFERENCES

Clark, A. E. (1952). *J. Path. Bact.*, **64**, 423.
Custer, R. P. (1933). *Amer. J. med. Sci.*, **185**, 617.
Dacie, J. V., and White, J. C. (1949). *Journal of Clinical Pathology*, **2**, 1.
Dameshek, W., Henstell, H. H., and Valentine, E. H. (1937). *Ann. intern. Med.*, **11**, 801.
Reich, C., Swirsky, M., and Smith, D. (1944). *J. Lab. clin. Med.*, **29**, 508.
White, J. C., Baker, J. R., and Griffin, J. G. (1946). *J. Path. Bact.*, **58**, 155.

J. clin. Path. (1952), 5, 250.

VITAMIN B_{12} ASSAY IN BODY FLUIDS USING *EUGLENA GRACILIS*

BY

G. I. M. ROSS*

From the Department of Pathology, Postgraduate Medical School of London

(RECEIVED FOR PUBLICATION SEPTEMBER 28, 1951)

The methods of assay of vitamin B_{12} most generally used depend on the fact that some lactobacilli require this vitamin as an essential growth factor and that growth is proportional to the amount available over a certain range of concentration. Some desoxynucleotides (Shive, Sibley, and Rogers, 1951), desoxyribonucleosides and thymidine particularly, and many other substances too, have, however, been found to replace vitamin B_{12}, although in relatively large amounts only. Assays with lactobacilli then are not entirely specific and this fact, combined with rather low sensitivity, makes these methods unsatisfactory for detecting the low concentrations of vitamin B_{12} found in some body fluids. The results of lactobacillus assays of whole blood of a number of animal species (Couch, Olcese, Witten, and Colby, 1950), of milk of a number of animal species (Collins, Harper, Schreiber, and Elvehjem, 1951), of urine of subjects receiving vitamin B_{12} therapy (Chow, Lang, Davis, Conley, and Ellicott, 1950), and of human urine (Girdwood, 1951) have, however, been reported. Plate assays with a *Bact. coli* mutant requiring vitamin B_{12} as a growth factor (Bessell, Harrison, and Lees, 1950) are also lacking in sensitivity, but, because of the simplicity of the method and rapidity of growth, are useful for the assay of large concentrations such as are found in urine after injection of vitamin B_{12} (Chesterman, Cuthbertson, and Pegler, 1951). Chemical methods of assay are being developed (Boxer and Rickards, 1951; Fantes, Ireland, and Green, 1950), but are still insufficiently sensitive to detect concentrations normally found in body fluids.

The use of an alga, *Euglena gracilis* var. *bacillaris*, for the assay of vitamin B_{12}, crystalline and in liver extract, was described by Hutner, Provasoli, Stokstad, Hoffmann, Belt, Franklin, and Jukes (1949). Assays with the Euglena by both the tube and plate method of a variety of material including plants, earth, pond water, and milk have also been reported (Robbins, Hervey, and Stebbins, 1950a, b, 1951). Preliminary observations made in assays of serum, urine, and cerebrospinal fluid with the Euglena have been previously described (Ross, 1950).

Crystalline vitamin B_{12}, B_{12a}, and B_{12c}, and liver extracts produce a quantitative, and almost equivalent, growth response of the test organism, and the body fluids which have been tested show similar activity. No other substance has yet been shown to replace vitamin B_{12} in the growth of the Euglena, and in this work it has been assumed that the Euglena growth-promoting effects of body fluids are, in fact, due to some form of the vitamin, and values in assays of these fluids are expressed as equivalents of vitamin B_{12}.

The present paper describes a technique of assay of serum and urine, which is also applicable to such material as liver extracts and liquid culture media.

Method

Quantitative assays have been made by comparing the growth-promoting effects of varied dilutions of the test fluids with those of known concentrations of crystalline vitamin B_{12}.

Dilutions in distilled water of test fluids and standards are added to test-tubes. Volumes are made up to 2 ml. with distilled water and 2 ml. of double-strength basal medium are added to give a final volume of 4 ml. Assay batches conveniently contain 200–250 tubes and include a complete set of standards and six control tubes with no vitamin B_{12}. The tubes are then heated in a water bath at 100° C. for 15 minutes and, after cooling, inoculation is made with one drop of a dense, actively growing stock culture of the Euglena, usually five to seven days old. The tubes are then incubated at 30° C. for eight to 10 days.

Heating at 100° C. for 15 minutes is essential to liberate the maximum amount of vitamin B_{12} from the heat-labile complex in which it may be found in

* In receipt of a grant from the Medical Research Council.

serum (Ross, 1950), and is effective in killing contaminating fungi.

An estimate of uncombined vitamin B_{12} in these fluids can be made by setting up tubes, either unheated, or heated at only 56° C. for 30 minutes, a temperature which releases little vitamin B_{12} from combination, yet helps to prevent contaminating growth.

Apparatus.—Test-tubes, 6 × ⅝ in., plugged with cotton-wool, are used for assays, and 6 × ¾ in. tubes for maintenance of stock cultures.

The optimum temperature for the Euglena is 28–31° C. with inhibition effects at about 32° C. (Hutner *et al.*, 1949). The organism is rapidly killed at temperatures higher than 40° C. A perspex water tank (2 ft. 6 in.×1 ft. 3 in.×7 in.) heated by a test-tube immersion coil element and held at 30° C. by a Sunvic thermostat is used for incubation. An electric motor-driven water stirrer in a corner near the heating element circulates the water, and the temperature is found generally to vary from 30° C. by no more than 0.5° C. from one end of the bath to the other. Illumination is supplied from below by two 3 ft. 40-watt fluorescent strip lights which, by extending beyond the ends of the bath, maintain uniform brightness. Tubes are either bundled together at random with elastic bands or packed in rectangular partitions made of perspex. Racks are less economical of space.

A photoelectric colorimeter with a 1 cm. deep rectangular cell is used to measure turbidity of cultures.

Basal Medium.—The basal medium used is that recommended by Hutner, Provasoli, Schatz, and Haskins (1950), but with slightly different proportions of the metals. The exact amounts given in that paper and the use of $Ca(NO_3)_2$ in place of $CaCO_3$ have not been tried, but the differences in growth effect are unlikely to be significant. Hutner *et al.* (1949) found that thiamine (aneurine), although in no way replacing vitamin B_{12}, is a second essential growth factor and must be present in at least 2 mμg./ml. concentration. This amount is greatly exceeded in the medium used. The composition (double strength) is as shown in the table below.

Potassium dihydrogen phosphate (KH_2PO_4) 5% ..	20 ml.
l-glutamic acid	10 g.
dl-malic acid	2 ,,
Magnesium sulphate ($MgSO_4.7H_2O$) 5%	20 ml.
Zinc sulphate ($ZnSO_4.7H_2O$) 8·8%	1 ,,
Calcium carbonate ($CaCO_3$) 1%	10 ,,
Ferrous sulphate ($FeSO_4.7H_2O$) 0·3%	10 ,,
Manganese sulphate ($MnSO_4.H_2O$) 1·23%	1 ,,
or ($MnSO_4.4H_2O$) 1·62%	1 ,,
Cupric sulphate ($CuSO_4.5H_2O$) 3·92%	0·1 ,,
Cobalt sulphate ($CoSO_4.7H_2O$) 1·73%	0·1 ,,
Sodium molybdate ($Na_2MoO_4.2H_2O$) 1·01% ..	0·1 ,,
Boric acid (H_3BO_3) 1·41%	2 ,,
Aneurine hydrochloride	2 mg.
Water to	1,000 ml.

The ingredients are steamed to dissolve, and the *p*H adjusted to 3.6 approximately. The mixture is filtered, bottled, and steamed to sterilize.

The concentration of the basal medium has proved satisfactory, equal parts of double-strength medium and of vitamin B_{12} in distilled water giving optimum growth.

Maintenance of the medium *p*H at about 3.6 prevents most bacterial growth, but does not prevent growth of fungi which are particularly favoured by the 30° C. temperature and long incubation. Provided all tubes are adequately heated and inoculated with care, contamination rarely occurs, although it has been troublesome in experiments without preliminary heating. Growth is good down to *p*H 2.5, but appears to be retarded considerably below this, and at this *p*H vitamin B_{12} loses activity at room temperature (Smith, 1951).

Stock Cultures of the Euglena.—These were maintained for 10 months by weekly subculture in 10 ml. volumes of single-strength basal medium with 50 μμg./ml. crystalline B_{12}, and at between *p*H 3 and *p*H 4. There was no development of vitamin B_{12} independent variants, although several hundred tubes with no vitamin B_{12} were inoculated in this time. There was, however, some change in growth characteristics after four to five months, during which period boron was not included in the medium. Growth was no longer as luxuriant and rapid as when the culture was first received, but with the addition of boric acid, growth improved, and the organism now appears to be stable in this respect. It is doubtful if this simple basal medium supplemented only by crystalline vitamin B_{12}, and kept at acid *p*H, can provide ideal conditions for reproduction. Cultures have also been maintained from time to time with liver extracts, as recommended by Hutner *et al.* (1949), but with no obvious difference in responsiveness to vitamin B_{12}. Cultures with 80 μμg./ml. vitamin B_{12} are now being used to increase inoculum density. Greater concentrations are not advised because of the increased carry-over of vitamin in the inoculum and because the resulting increase in density of growth leads to excessive alkali production and therefore to greater risk of bacterial contamination.

If a culture becomes contaminated it may be purified by growth on nutrient agar plates, supplemented with basal medium and vitamin B_{12}, to obtain separate colonies of the Euglena. Plates are incubated in the warm atmosphere of the water bath where light also assists growth.

Standards.—A crystalline vitamin B_{12} solution, either 20 μg./ml. or 1 μg./ml., is diluted to give final concentrations of 50, 25, 15, 10, 5, 2.5, and 1.25 μμg./ml. At least four tubes of each concentration are set up so that a set of seven standards requires a minimum of 28 tubes.

Stock standards certainly retain potency for one to two weeks, but show on average about a 10% loss in potency after a month at 4° C. They are, therefore, preferably prepared fresh at least every fortnight and set up together with some tubes of the previous standards for comparison.

Material for Assay

Body fluids are kept frozen until tested, and, as activity may decline on keeping, assays should be made if possible not more than three days after collection of the specimen.

As quantities of serum available for assays are usually limited, it is economical and time-saving to vary dilutions according to the probable vitamin B_{12} concentration. All tests are preferably set up in more than one dilution with at least five tubes for each dilution, but, when large numbers of tests of sera and urines are being set up, it has been convenient to use only one dilution (e.g., 1 in 40, which allows the assay of material with between 50 and 2,000 $\mu\mu$g./ml.). When low values are expected, as with serum and urine from patients with pernicious anaemia in relapse, dilutions in the range 1 in 8 to 1 in 20 are used. Serum from patients with a slightly higher level (e.g., cases of pernicious anaemia on fortnightly or monthly maintenance injections of B_{12} or liver and taken towards the end of the interval between injections) may require to be diluted 1 in 20 and 1 in 40. When high values are likely, as shortly after injection of vitamin B_{12}, urine and serum are set up at dilutions of 1 in 40, 1 in 400, and 1 in 4,000 or more. Liver extracts for assay require dilution to about 1 in 400,000 to 1 in 800,000.

Bacterial contamination of specimens must be avoided in view of the production of vitamin B_{12} by some organisms and of its removal from solution by others, particularly by some strains of *Bact. coli* (Davis and Mingiolo, 1950, confirmed by Hayes and the author, unpublished data). Ternberg and Eakin (1949) found that vitamin B_{12} in combination with a factor in gastric juice was not available to *Bact. coli*, but whether the serum factor–B_{12} combination behaves in a similar way has not yet been investigated. The activity of some urines stored at 4° C. has in fact been found to increase, presumably either from breakdown of previously inactive compounds or from bacterial synthesis. Addition of toluene has assisted preservation, but the volatile preservatives recommended by Hutner and Bjerknes (1948) may be more suitable for this purpose, provided 100° C. heating is carried out. Urine must be completely free from any contamination with faeces because of its high content of vitamin B_{12}.

Reading and Calculating Results

Growth is read by measuring turbidity in a photoelectric colorimeter, using a red filter, and is recorded as optical density. The 4 ml. final volume in each tube is convenient both for pipetting and for reading with the colorimeter.

Vigorous shaking is required to disperse clumps of algae, and tubes with serum froth markedly. Bubbles are dispersed immediately by the addition of one drop of a caprylic alcohol-water (1 in 5) mixture. This should not be added before vigorous shaking, because of the occasional fragmentation of algae and undue cloudiness which may result.

The more concentrated the serum, the greater the turbidity due to serum proteins. At the acid *p*H of the basal medium there is little tendency for precipitation even with 100° C. heating, but, as the isoelectric point of the protein is approached, turbidity increases. The *p*H is only very slightly altered by the addition of dilutions of serum, but with profuse growth of the Euglena, alkali is produced which may raise the *p*H above *p*H 5 (the pK of methyl red). This degree of alkalinity is produced by the Euglena in the presence of an aqueous concentration of 50 $\mu\mu$g. per ml. of vitamin B_{12}. The alkali formed by the Euglena is proportional to the turbidity of the culture and therefore to the amount of vitamin B_{12} available for growth (Fig. 1). Tubes with sera diluted 1 in 32 or less, haemolysed sera, concentrated urines, bile, etc., may show colour or turbidity due to the test fluid itself which can be read in the supernatant after the Euglena cells have all sedimented,

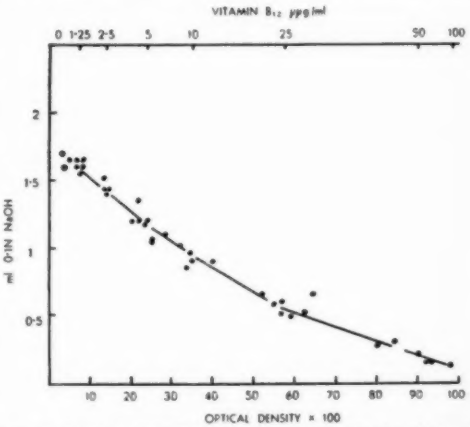


FIG. 1.—Titration of alkali produced by growth of Euglena in 4 ml. volumes with vitamin B_{12} standards (1·25, 2·5, 5, 10, 25, 50, 100 $\mu\mu$g./ml.) is shown, using phenolphthalein as indicator. With 1·25 $\mu\mu$g./ml. B_{12} there is little growth, so that 1·6 ml. 0·1 N NaOH is required to raise *p*H from 3·6 to 9·7, while with 50 $\mu\mu$g./ml. B_{12} there is profuse growth and only 0·15 ml. 0·1 N NaOH is required. Alkali production by the Euglena is proportional to amount of growth and therefore to vitamin B_{12} available.

being immobilized in the presence of caprylic alcohol. This blank reading is subtracted from the total turbidity, and the vitamin B_{12} concentration is then calculated by comparison of this figure with those found with the standard solutions, which are plotted on log x mm. and cm. graph paper giving the type of curve shown in Fig. 2. Concentrations

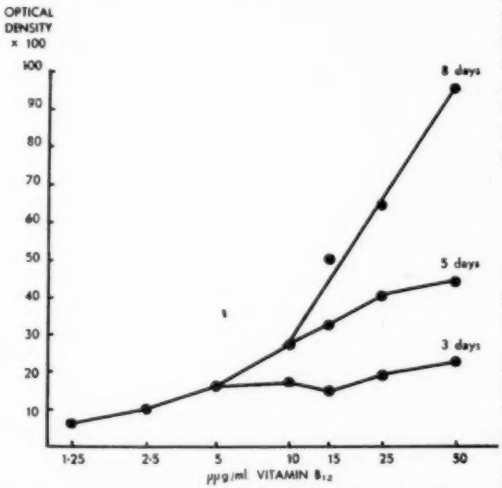


FIG. 2.—Growth of *Euglena gracilis* in presence of seven concentrations of crystalline vitamin B_{12} showing the need of long incubation to give differentiation between growth with varied strengths of vitamin. Each point corresponds to an average reading of three tubes.

normally found in body fluids are conveniently measured in $\mu\mu$g./ml.

Control tube readings vary from batch to batch, particularly as the result of difference in inoculum size and carry-over of unabsorbed vitamin B_{12} in each drop of inoculum. Average optical density readings are from 0.02 to 0.06, but may be slightly higher. Standard readings also vary between batches, and therefore sets of standards must always be included.

It should be noted that incubation for as long as 10 days may be necessary to allow differentiation of growth with higher concentrations of vitamin B_{12}. Growth continues until all available vitamin B_{12} has been used up, or until, with 150 $\mu\mu$g./ml. or more, growth reaches the maximum concentration of about 5 to 7 million cells per ml. (Hutner *et al.*, 1950; Robbins *et al.*, 1950b). Absorption of vitamin B_{12} in 50 $\mu\mu$g./ml. concentration is rapid, and within two days of inoculation no vitamin B_{12} is detectable in the supernatant, indicating that cells absorb much more than their immediate requirements and continue to divide using this store. The vitamin B_{12} requirement for the formation of each Euglena cell has been stated to be about 4,900 molecules (Hutner *et al.*, 1950) and about 7,100 molecules (Robbins *et al.*, 1950b). Figures of from 6,000 to 7,000 have been found by the author using varying initial concentrations of vitamin B_{12} and taking the molecular weight as 1300. Slight variation in requirement is likely in view of the difference in cell size noted from time to time by Robbins *et al.* (1950b). Heating at 100° C. does not liberate vitamin B_{12} from the Euglena cell, indicating, as expected, a radical alteration of the molecule in metabolism.

Sensitivity, Accuracy, and Specificity of the Method

Sensitivity.—While the sensitive range of assays with lactobacilli and *Bact. coli* lies above 50 $\mu\mu$g./ml., that of the Euglena method is from 5 to 25 $\mu\mu$g./ml. and as little as 1 $\mu\mu$g./ml. can be detected.

In body fluids, however, it is not practicable to detect less than about 10 $\mu\mu$g./ml., as serum present in greater concentration than 1 in 8 coagulates with heating at 100° C. and may be inhibitory to growth. Urine likewise may be inhibitory at 1 in 10 or 1 in 20 so that a concentration of less than 10 to 20 $\mu\mu$g./ml. cannot be detected. Cerebrospinal fluid, partly because of its low protein content, can be set up in higher concentration than serum; 1 in 4 has not been inhibitory. Whole blood, milk, and bile produce a turbid or coloured solution which is unsuitable for direct assay, particularly because of the resulting interference with illumination of the alga.

Accuracy.—The definite molecular vitamin requirement of each cell, already mentioned, makes the number of cells formed with sufficient time for full growth and the resulting turbidity with standard solutions of vitamin B_{12} very consistent from batch to batch. With the more complex fluids such as serum, urine, and gastric juice, results are also consistent when assays are made on fresh material. When test fluids, for example serum and gastric juice, contain substances capable of combining with vitamin B_{12}, the extent to which the vitamin and its fractions may be readsorbed and thus reinactivated after heating will influence the true assay value unless the combining power can be destroyed. Several tests of serum and gastric juice, however, indicate that variation of time of heating at 100° C. does not greatly alter the result within the first hour, but after that the activity of the vitamin is reduced. There is a much

quicker liberation of vitamin B_{12} when heating is carried out *after* the addition of basal medium at *p*H 3.6 than when heating is done at *p*H 7.5 *before* the addition of basal medium (Figs. 3 and 4). Pooled normal serum, and pooled sera from patients with pernicious anaemia receiving maintenance doses of vitamin B_{12}, did not differ in their power of binding of vitamin B_{12}. The optimum time at 100° C. in routine assays where basal medium is added before heating is about 15 minutes. The extent of recovery of known amounts of the varieties of vitamin B_{12} added to such fluids is usually almost complete.

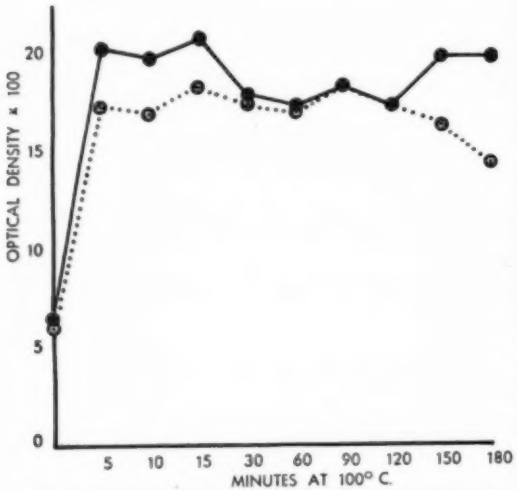


FIG. 3.—The effect of time of heating at 100° C. of normal serum (●) and of serum from a patient with pernicious anaemia during treatment with vitamin B_{12} (○) is shown. The basal medium (*p*H 3.6) was added *before* heating. Release of vitamin B_{12} is rapid—maximum at five to 15 minutes. Each point represents average reading of four tubes.

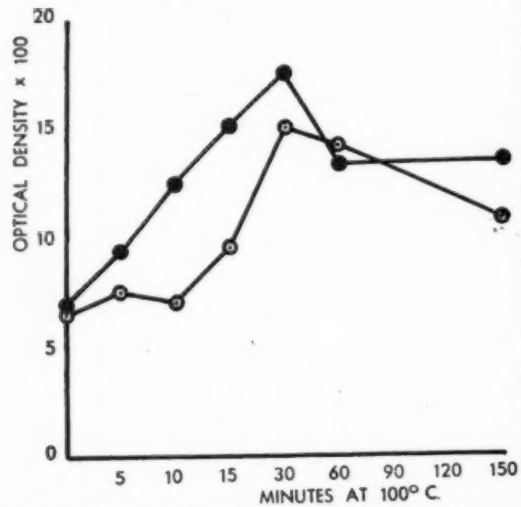


FIG. 4.—The effect of time of heating at 100° C. of normal serum (●) and of serum from a patient with pernicious anaemia during treatment with vitamin B_{12} (○) in water at *p*H 7.5 is shown. The acid basal medium was added *after* heating. Release of vitamin B_{12} is slow—maximum at 30 minutes. Each point represents average reading of four tubes.

Specificity.—As mentioned in the introduction, no substance has yet been identified which can replace vitamin B_{12} in the metabolism of the Euglena cell. Hutner *et al.* (1949) showed that nicotinic acid, pantothenic acid, pyridoxine HCl, biotin, pteroylglutamic acid, *p*-aminobenzoic acid, and thymidine were without significant effect on the growth response, either alone or in combination with vitamin B_{12} in the concentrations with which the tests were made. These results have been confirmed where they have been repeated as shown below and some additional substances have been tested.

Cysteine hydrochloride, nicotinic acid, pantothenic acid plus glycine, pteroylglutamic acid plus *p*-aminobenzoic acid, and methionine were added in final concentrations of 50, 5, 0.5, and 0.05 μg./ml., with and without vitamin B_{12}, 5 μμg./ml. Nicotinic acid, 50 μg./ml., was completely inhibitory, while 5 μg./ml. was not. Pteroylglutamic acid, *p*-aminobenzoic acid, pyridoxine hydrochloride, glycine, pantothenic acid, and uracil in 0.5 μg./ml. concentration without vitamin B_{12} and in 50, 5, 0.5, 0.05 μg./ml. concentrations with vitamin B_{12} 5 μμg./ml. were also tested. Ethyl alcohol, 12.5%, 2.5%, 0.25%, 0.025%, 0.0025%, and ammonium hydroxide, 5%, 0.5%, 0.05%, and 0.005%, with and without vitamin B_{12} 5 μμg./ml., were tested because of their use for dissolving some of the substances listed above. Ethyl alcohol, 12.5%, but not 2.5%, and 5% ammonium hydroxide, but not 0.5%, were inhibitory. Leuconostoc citrovorum factor (folinic acid), 75 μg./ml., also did not replace vitamin B_{12}.

Although some variation in amount of growth was found with certain concentrations of some of these substances in the presence of vitamin B_{12}, there was no evidence of substitution in its absence. When pantothenic acid and glycine in final concentrations of both 250 and 25 μg./ml., alone and together, were added to normal serum diluted 1 in 40, no significant alteration in growth response was found. Both normal sera and sera from patients with pernicious anaemia, however, have about 20% greater activity when heated in

the presence of 0.05% ascorbic acid. Inclusion of ascorbic acid at the above concentration in the standard tubes containing vitamin B_{12} in distilled water, however, has decreased growth so that fair comparison cannot be made by including ascorbic acid in all tubes of an assay batch. Ascorbic acid, 0.005%, with vitamin B_{12} and distilled water was not inhibitory. Sulphathiazole, penicillin, streptomycin, aureomycin, chloramphenicol, and *para*-aminosalicylic acid when added in concentrations likely to be found in human serum during treatment with these drugs were also without significant effect on growth of the alga.

Comparative bioautography of vitamin B_{12} and related factors using *Euglena gracilis* and *L. leichmannii* has shown that the Euglena responds only in the area of the slower moving factors where vitamin B_{12} is expected to appear and does not respond to pure desoxyribonucleic acid or to similar substances in both liver extract and corn steep liquor which stimulate growth of the lactobacillus (Picken and Bauriedel, 1950).

A wide variety of substances has also been tested by Robbins *et al.* (1950b) using *Euglena gracilis*, mainly in plate assay, and none was found to replace vitamin B_{12}.

The only substance which has yet been claimed to have any activity in replacing vitamin B_{12} for the Euglena is 1, 2, dimethyl-4, 5-diaminobenzene which may be contained within the vitamin B_{12} molecule. Its action, however, was incomplete because of its low solubility and a concentration one half to one million times that of the equivalent amount of vitamin B_{12} was required (Woolley, 1951).

It has not yet, however, been established that all the material in body fluids stimulating growth of the Euglena is haemopoietically active. In order to do this it will be necessary to assess the changes in both free and combined serum vitamin B_{12}-equivalent, in comparison with the changes in peripheral blood and bone marrow of patients with pernicious anaemia under treatment (Mollin and Ross, 1952). In spite of this reservation, preliminary results indicate that the method is of value in investigating megaloblastic anaemias and in following the absorption and excretion of the vitamin.

Concentrations in Body Fluids

Concentrations stated below as "total" indicate the amounts of vitamin B_{12} equivalent detected after 100° C. heating for 15 minutes; as "uncombined" (free) after 56° C. heating for 30 minutes; and as "combined," the difference between "total" and "uncombined."

The total vitamin B_{12} equivalents found by Euglena assay have already been reported as from 350 to more than 1,000 $\mu\mu$g./ml. in normal human serum, as up to 16 $\mu\mu$g./ml. in cerebrospinal fluid, and as up to 200 $\mu\mu$g./ml. in urine, while sera from four patients with pernicious anaemia had much lower concentrations than are normally found (Ross, 1950). Further observations have extended the range of values found in both normal human serum and urine. Details of these results were given in a recent paper (Mollin and Ross, 1952).

Human ascitic and pleural fluid, and gastric juice from subjects without megaloblastic anaemia, when heated at 100° C. allowed growth comparable to that found with normal human serum. Part of the activity of the first two fluids has been combined and part free, but most specimens of gastric juice have had no uncombined activity. One specimen of knee-joint fluid and of acromial bursar fluid with high protein content had no uncombined activity and a combined equivalent of 150 $\mu\mu$g./ml. Human seminal fluid and plasma (oxalated, citrated, or heparinized) gave results comparable to serum.

Guinea-pig, horse, and rabbit serum have also been assayed with the Euglena. Samples from these three species have had variable concentrations ranging from 200 to 4,000 $\mu\mu$g./ml., but rabbit serum has shown particularly high concentrations. One rabbit serum had the equivalent in $\mu\mu$g./ml. vitamin B_{12} of 36,000 total and 29,000 uncombined and another had 50,000 total and 40,000 uncombined.

The vitamin B_{12} equivalents in sera then vary considerably even within species, and, in human sera on which the majority of observations has been made, this variation is mainly of the uncombined substance, while combined values remain much more constant. Combined values rarely exceed 500 $\mu\mu$g./ml. in normal serum, so that higher total concentrations are mainly due to free material, suggesting either that combining power of serum is limited or that the material in circulation is not readily able to be bound. Both the combined and free material, like pure vitamin B_{12}, is microbiologically inactivated by 100° C. heating for one hour at *p*H 12. The significance of this uncombined material is more fully discussed elsewhere (Mollin and Ross, 1952).

Fresh, 24-hour collections of urine from normal subjects contain readily detectable amounts of the

vitamin, although occasional very dilute samples are inactive. Heating of urine at 100° C. has increased the yield of vitamin by only 0 to 10%, suggesting that there is a lack of combining substance in urine.

There are as yet few reports of the concentrations of vitamin B_{12} in body fluids. The presence of a *Euglena gracilis* vitamin in urine was originally demonstrated by Hutner (1936). *L. leichmannii* assays of whole blood gave a range of from 600 to 1,400 $\mu\mu$g./ml. in 10 normal human subjects and of from 400 to 15,000 $\mu\mu$g./ml. in 15 different animal species (Couch *et al.*, 1950). Of these animals tested, rabbit blood had the greatest amounts, although these were not so high as two of those found by Euglena assay and reported above. *L. leichmannii* assays of urine have also been reported. Chow *et al.* (1950) and Chow (1951) were unable to detect the vitamin in normal urine, but Girdwood (1951) has given the results of such assays on the urine of normal subjects, and of patients with sprue syndrome and with pernicious anaemia.

Summary

A method of assay of vitamin B_{12} equivalent in body fluids using *Euglena gracilis* var. *bacillaris* is described. This microbiological method is the most sensitive available, detecting as little as 1 $\mu\mu$g./ml. and giving greatest accuracy in the range of from 5 to 25 $\mu\mu$g./ml.

Material in body fluids stimulating growth of the Euglena may be "combined" or "uncombined." The maximum amount of combined material is detected after 100° C. heating at *p*H 3.6 for 15 minutes, and the vitamin is more rapidly freed from combination in serum when heated after addition of the acid basal medium than when heated before such addition. The uncombined vitamin is detected without such preliminary heating.

Human plasma, pleural, ascitic, seminal, bursa and joint fluid, and gastric juice assayed with the Euglena contained amounts similar to those found in normal human serum.

A number of substances, including amino-acids, vitamins, and antibiotics, were tested by Euglena assay and were not found to replace vitamin B_{12}.

I am grateful to Professor Lord Stamp for advice and encouragement, to Dr. D. L. Mollin for his co-operation, to Dr. W. F. J. Cuthbertson for advice and for the strain of *Euglena gracilis*, and to Glaxo Laboratories, Ltd., for the various types of vitamin B_{12} used. Miss J. Davenport has given valuable assistance in the assays.

References

Bessell, C. J., Harrison, E., and Lees, K. A. (1950). *Chem. and Ind.*, 561.
Boxer, G. E., and Rickards, J. C. (1951). *Arch. Biochem.*, **30**, 392.
Chesterman, D. C., Cuthbertson, W. F. J., and Pegler, H. F. (1951). *Biochem. J.*, **48**, 51.
Chow, B. F. (1951). *J. Nutr.*, **43**, 323.
—— Lang, C. A., Davis, R., Conley, C. L., and Ellicott, C. E. (1950). *Bull. Johns Hopk. Hosp.*, **87**, 156.
Collins, R. A., Harper, A. E., Schreiber, M., and Elvehjem, C. A. (1951). *J. Nutr.*, **43**, 313.
Couch, J. R., Olcese, O., Witten, P. W., and Colby, R. W. (1950). *Amer. J. Physiol.*, **163**, 77.
Davis, B. D., and Mingioli, E. S. (1950). *J. Bact.*, **60**, 17.
Fantes, K. H., Ireland, D. M., and Green, N. (1950). *Biochem. J* **46**, 34.
Girdwood, R. H. (1951). *Edinb. med. J.*, **58**, 309.
Hutner, S. H. (1936). *Arch. Protistenk.*, **88**, 93.
—— and Bjerknes, C. A. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 393.
—— Provasoli, L., Stokstad, E. L. R., Hoffmann, C. E., Belt, M., Franklin, A. L., and Jukes, T. H. (1949). Ibid., **70**, 118.
—— —— Schatz, A., and Haskins, C. P. (1950). *Proc. Amer. phil. Soc.*, **94**, 152.
Mollin, D. L., and Ross, G. I. M. (1952). *Journal of Clinical Pathology*, **5**, 129.
Picken, J. C., and Bauriedel, W. R. (1950). *Proc. Soc. exp. Biol., N.Y.*, **75**, 511.
Robbins, W. J., Hervey, A., and Stebbins, M. E. (1950a). *Science* **112**, 455.
—— —— —— (1950b). *Bull. Torrey bot. Cl.*, **77**, 423.
—— —— —— (1951). Ibid., **78**, 363.
Ross, G. I. M. (1950). *Nature, Lond.*, **166**, 270.
Shive, W., Sibley, M. E., and Rogers, L. L. (1951). *J. Amer. chem. Soc.*, **73**, 867.
Smith, E. L. (1951). *Nutr. Abstr. Rev.*, **20**, 795.
Ternberg, J. L., and Eakin, R. E. (1949). *J. Amer. chem. Soc.*, **71**, 3858.
Woolley, D. W. (1951). *J. exp. Med.*, **93**, 13.

J. clin. Path. (1952), **5**, 257.

THE CONSTRUCTION AND CLINICAL SIGNIFICANCE OF THE COAGULOHEPATOGRAM*

BY

W. RIEBEN

From the Laboratory of the Division of Surgery, University of Lausanne, Switzerland

(RECEIVED FOR PUBLICATION MAY 8, 1951)

The term coagulohepatogram is proposed for curves obtained with a simple two-stage prothrombin method by successive determinations of thrombin formed from prothrombin during a 15-minute period from the start of activation with thromboplastin-calcium. The three liver specific factors which comprise the coagulohepatogram are (1) accelerator, (2) prothrombin, and (3) the natural plasma antithrombin, and these can be simultaneously measured by using three dilutions of plasma 1:25, 1:50, and 1:75.

Technique for the Determination of Plasma Prothrombin by Two-stage Method

For the determination of plasma prothrombin levels, two main types of methods have been chiefly employed. Quick (1938, 1945) introduced the first satisfactory one-stage procedure. Within a short time after its original publication Warner, Brinkhous, and Smith (1936) described a more complicated procedure in which the whole clotting process is separated into two phases.

In recent years many observations have been made that indicate that the two types of methods do not necessarily measure one and the same plasma components. Such discrepancies are found in liver diseases (Ziffren, Owen, Warner, and Peterson, 1942), pernicious anaemia (Warner and Owen, 1942), in the behaviour of plasma of newborns and infants (Brinkhous, Smith, and Warner, 1937), and of banked blood and lyophile plasma. Differences in appraising the effect of methylxanthines have also been described (Rieben, 1946a).

The original two-stage method of Warner *et al.* (1936) is based on serial dilutions of plasma before transformation of the prothrombin into thrombin by means of thromboplastin and calcium. Greater accuracy has been claimed for it than for the one-stage procedures, but it is too elaborate for clinical use.

The present contribution concerns an adaptation of the two-stage method of Herbert (1940) which is capable of great accuracy and which has proved to be extremely satisfactory for experimental purposes and routine use on a large scale. As many as 25 plasmas can be analysed by one person in a day. The procedure is based on readily available preparations and is much simpler than the Herbert technique. As a rule only one plasma dilution is tested, except for coagulohepatograms, where three different dilutions are recommended.

Variations in the concentration of accelerator factors, termed "accelerator" in this paper, seriously affect the values obtained with one-stage procedures.

This technique was devised in the Department of Medicine, New York Hospital, and Cornell University Medical College, New York.

Reagents.—The following are needed.

Thromboplastin.—Two solutions are prepared: solution 1, 0.9% NaCl; solution 2, 0.25 g. anhydrous calcium chloride and 4.28 g. of NaCl are dissolved in distilled water and made up to 500 ml. To 150 mg. of dried rabbit brain ("bacto" thromboplastin†) in a 50 ml. conical centrifuge tube are gradually added 15 ml. of saline with continuous grinding in order to obtain a smooth emulsion. The tube is then transferred to a beaker containing water at 54° C., and the contents are stirred for at least 10 minutes with a glass rod to destroy prothrombin and antithrombin. After cooling to room temperature 15 ml. of the calcium-containing saline (solution 2) are added. After continuous stirring for at least five minutes, the contents of the tube are centrifuged for five minutes at 2,000 r.p.m. The supernatant fluid is pipetted off. It can be diluted up to one half of its quantity with a solution made up from identical amounts of solution 1 and solution 2.

* Abstract of a paper read at the Third International Congress of Haematology, Cambridge, in August, 1950.

† Manufactured by Difco Laboratories, Detroit, Mich., U.S.A.

Fibrinogen.—It was found advantageous to use a stable dried fraction of beef plasma commercially obtainable from the Armour Laboratories, Chicago, Illinois. To obtain 50 ml. of a buffered fibrinogen solution, 0.5 mg. of fibrinogen is weighed out and transferred through a wide-mouth funnel to a seamless cellulose dialysis tube of 30 mm. diameter. Then 50 ml. of buffered oxalated saline are pipetted into the sac and the contents are dialysed for one hour against 2 litres of the same buffered oxalated saline in a 2-litre graduated cylinder. The fibrinogen sack is then transferred to a similar cylinder containing buffered saline only. Dialysis is continued for another hour. The buffered fibrinogen is then placed in 100 × 13 mm. pyrex tubes and kept frozen in the freezing compartment of a refrigerator. Before use the amount needed for each series is thawed out at 4–7° C. Preparations stored in the frozen state keep for at least two or three weeks. They are standardized simultaneously against one or more plasma of normal prothrombin activity, or against a thrombin standard as indicated later.

Veronal Buffer Solution.—Instead of imidazol, M/10 sodium veronal-N/10 HCl buffer is recommended. The buffer mixture described here tends to crystallize when stored for prolonged periods of time. For this reason it has to be made up at frequent intervals from stock solutions of M/10 sodium-diethyl-barbiturate and N/10 HCl. In order to obtain 100 ml. of this buffer giving a *p*H of 7.6, 61.5 ml. of M/10 sodium veronal and 38.5 ml. of N/10 HCl are mixed.

Buffered Oxalated Saline.—To 40 ml. of *p*H 7.6 veronal buffer are added 40 ml. of 1.85% potassium oxalate and the mixture diluted to 2 litres with 0.9% saline.

Buffered Saline.—To 40 ml. of *p*H 7.6 veronal buffer isotonic saline is added to make a final volume of 2 litres.

Veronal Buffer for Daily Determinations.—By adding 3.85 ml. of N/10 HCl to 6.15 ml. of M/10 sodium veronal a buffer of *p*H 7.6 is made which is used in preparing the activation mixture with equal amounts of (1:50) plasma dilutions with saline and double the amount of thromboplastin calcium mixture.

Procedure.—This is as follows:

Blood Collection.—Into a sterilized and dry 2 ml. syringe are drawn up 0.2 ml. of sterilized 1.85% potassium oxalate (M/10). From the cubital vein enough blood is now withdrawn to bring it up to the 2 ml. level in the syringe. After mixing, the needle is removed and the contents gently transferred to a 15 ml. pyrex centrifuge tube which is then centrifuged for seven or eight minutes at 2,500 r.p.m. The plasma is separated and at once transferred to a 100 × 13 mm. pyrex tube, and 0.1 ml. portions diluted with a 5.0 ml. portion of saline (for coagulohepatogram dilute also with 2.5 and 7.5 ml. saline respectively). The diluted plasma is stored at 4–7° C. It should be analysed within two to five hours.

Conversion of Prothrombin into Thrombin.—During the first stage of the determination the dilute plasma is converted to yield thrombin by addition of thromboplastin and calcium. Of the freshly prepared *p*H 7.6 veronal buffer, 0.5 ml. is added to 0.5 ml. of the 1:50 plasma dilution and 1 ml. of thromboplastin-calcium mixture then added. Using 1:50 dilutions, prothrombin times between 24 and 40 seconds were obtained for normal plasmas. For plasmas of very low prothrombin activity 1:25 dilutions can be used both for unknown and control plasma.

Determination of Thrombin Activity.—In the second stage fibrinogen is added to an aliquot of the activation mixture and the activity of the thrombin formed is determined by measuring the time required to form a fibrin clot. At room temperature a point close to maximum thrombin activity is normally reached in two to five minutes. During incubation the tube is taken out after the first two minutes and the open end held close to an indirect light source (to recognize the Tyndall phenomenon). The contents are gently shaken at half-minute intervals by swinging the lower end of the tube in a circle until a fine fibrin web appears, indicating a point close to maximum thrombin activity. At this moment 0.2 ml. of the mixture is pipetted into a preheated tube (37° C.). It is then left at 37° C. for 30 to 40 seconds and 0.1 ml. of fibrinogen solution is added from a 0.2 ml. pipette. This is best performed by resting the tube on the button of a stopwatch holder. The very instant the fibrinogen is blown out, the stopwatch is started by simultaneously exerting downward pressure with the tube.

After a five-second interval used for mixing the tube is again immersed in the 37° C. constant temperature water bath and the clotting time is accurately determined. This can be done by placing it on the horizontal glass portion of a Fisher Kahn viewer* under the magnifying lens.

After determining the clotting time for the first sample, two additional 0.2 ml. aliquots are tested in a similar fashion. The average of the three readings is used for the calculation of the clotting time.

Calculation.—The prothrombin activity of an unknown is obtained by dividing the average prothrombin time of a series of normal plasmas by the prothrombin time of the unknown and multiplying by 100. For example, 1:50 plasma dilutions of five different normal persons yielded prothrombin times of 31.3, 27.7, 34.3, 35.7, and 39.2 seconds respectively, the average being 33.6 seconds. The prothrombin time of a 1:50 dilution of an unknown was found to be 47.8 seconds. Its prothrombin activity is therefore 70.3%.

Error of the Method.—Mean and standard deviation, $\sqrt{\Sigma_d^2/(N-1)}$, were determined independently by two observers on 0.1 ml. plasma aliquots of 47 different plasmas. The mean errors at various levels were all related to a prothrombin activity of 100%. A mean deviation of ±1.55% and a standard deviation of ±1.88% were found in this large series.

* Fisher Scientific Co., Pittsburg, Pa., U.S.A.

Technique for Obtaining Coagulohepatograms

This has been devised for the simultaneous determination of accelerator and of prothrombin and antithrombin activity of blood plasma.

For the determination of coagulohepatograms a temperature of 37° C. is chosen. For the curves for the determination of accelerator activity a plasma dilution of 1: 75 is used. In this instance clotting times are determined every minute. The accurate determination of the interval from the start of activation to the moment of maximum activity is decisive, and for it I propose the term "accelerator time." Accelerator time is recorded in minutes and fractions of minutes and is compared with that of plasma of known accelerator activity.

For the determination of prothrombin activity the use of 1: 50 plasma dilutions is recommended and the procedure described above followed with one difference. Instead of carrying out the activation at room temperature, the tests are carried out at 37° C.

For the determination of antithrombin activity a plasma dilution of 1: 25 is chosen. Working at 37° C. a strong antithrombin effect can be observed and the thrombin units destroyed in 15 minutes can be read from the 1: 25 curve (Fig. 1).

It has been shown in a previous paper (Rieben, 1950) that for the accurate determination of antithrombin activity in low prothrombin plasmas, 1.5 units of thrombin in veronal-HCl-buffer of *p*H 7.6 is necessary in order to obtain a sufficiently long descending limb on the activation curve for determination of the antithrombin effect.

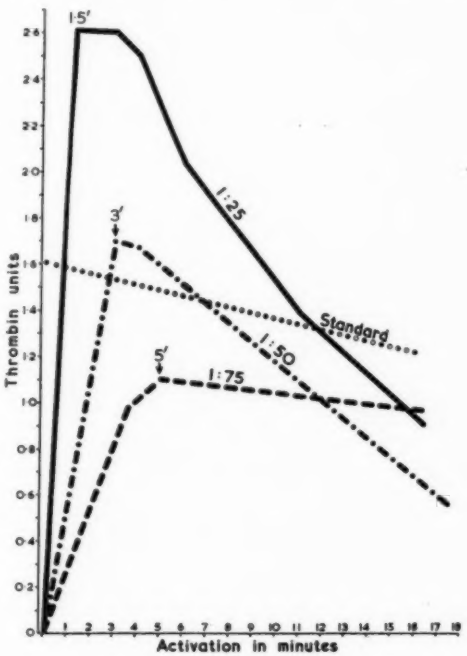


FIG. 1

For the determination of factor V methods have been described by Owren (1947), who used different dilutions of a standard plasma for comparison, and showed that "the velocity of thrombin formation rises with increasing concentration of factor V up to a certain limit. Further increase of factor V beyond this limit is without influence. No thrombin formation takes place without factor V."

Using 1: 75 dilutions of plasma, factor V present is sufficiently diluted to avoid falling into the range where further increase of accelerator does not any longer shorten the period of maximum activation.

The method of Ware and Seegers (1948) for the determination of Ac-globulin is based on a standardization procedure with known amounts of purified Ac-globulin. The authors admit that this method, though excellent theoretically, is very cumbersome, and is hardly applicable to clinical use. There is no reason why results obtained by either of the two procedures mentioned should not be useful when incorporated into coagulohepatograms.

The new method for accelerator activity determinations is the simplest clinical method known so far. It has the great advantage of making use of the same technique, the same reagents, and the same apparatus as the above described two-stage prothrombin method used by the author since 1945.

Reagents.—For coagulohepatograms the use of a thrombin standard is recommended. It permits quantitative appraisal of antithrombin activity and comparison of results obtained in different laboratories.

Fibrinogen.—While fibrinogen powder is readily available in Great Britain and the United States, it has only fairly recently become available on the Continent. When fresh it does not need dialysis and is ready for use by the addition of distilled water.

Thromboplastin.—"Bacto" thromboplastin has been used throughout. For the determination of accelerator activity variations of thromboplastin are of great importance. With a given prothrombin and accelerator activity the time used to obtain maximum thrombin depends on it. For this reason analysis of a plasma of known accelerator activity is simultaneously carried out with each series.

Thrombin.—Standardized crystallized thrombin (Hoffmann-La Roche, Bâle) was used throughout these experiments. The commercial preparation is available in 100 mg. amounts containing 60 international thrombin units (t.u.) per mg.

In order to standardize a daily series of coagulohepatograms, 5.0 mg. of crystallized thrombin are accurately weighed out and placed in a 50 ml. Erlenmeyer, and

immediately before use 18.75 ml. of chilled veronal HCl buffer are added. Of this, 1.0 ml. is rapidly transferred to a 15 ml. centrifuge tube containing 4 ml. of veronal-HCl-buffer. Of this final solution, 0.5 ml., representing 1.60 t.u., is pipetted into a 100 × 13 mm. pyrex tube kept in the constant temperature water bath at 37° C. This tube is then treated as those containing plasma dilution for prothrombin determinations.

Contrary to common belief, a diluted thrombin solution is sufficiently stable for 15 minutes in the presence of thromboplastin-calcium to make it an ideal standard for coagulohepatograms. The amount of thrombin per tube (1.60 t.u.) closely corresponds to a 1:50 dilution of plasma of 100% prothrombin activity (Table I).

TABLE I

VALUES AND RANGE OF ACTIVITIES OF COMPONENTS OF THE COAGULOHEPATOGRAM

Factor and Plasma Dilution	Maximum Thrombin Activity (Average)		Time Required for Maximum Activity* (min.)		Range of Activity (% of Average Normal)
			Average	Range	
	t.u.	a.t.u.			
1:25 plasma: Antithrombin	2.61	0·975	1·5	1·0–2·0	65–130
1:50 plasma: Prothrombin..	1·60	—	3·0	2·5–3·5	70–130
1:75 plasma: Accelerator ..	1·10	—	5	4–6	60–150

* Depends on activity of thromboplastin preparations, which vary from one laboratory to another.

Procedure.—This is as follows.

Blood Samples.—Due to the instability of accelerator, plasma separated immediately after venesection is kept at a temperature close to freezing. Instead of potassium oxalate suitable for prothrombin determination the use of 3.2% tribasic sodium citrate is recommended for coagulohepatograms.

Technique for Coagulohepatograms.—Plasma, 0.1 ml., is measured into three 10 or 15 ml. centrifuge tubes. By adding 2.5 ml., 5.0 ml., and 7.5 ml. of 0.9% saline, dilutions of 1:25, 1:50, and 1:75 are obtained. After mixing 0.5 ml. of each plasma dilution is placed in 100 × 13 mm. pyrex tubes in the 37° C. constant temperature water bath. After one minute 1.0 ml. of thromboplastin-calcium kept at 37° C. is added from a 1 ml. Mohr pipette. A first stopwatch is simultaneously started to measure activation time. At intervals of one to two minutes clotting times are determined on 0.2 ml. plasma aliquots by blowing 0.1 ml. of fibrinogen solution into the tubes and simultaneously starting a second stopwatch kept in a stopwatch holder. The procedure closely resembles that of the two-stage method for prothrombin determination described above. Instead of awaiting the appearance of a fibrin web, serial determinations are carried out during a 15-minute period to yield values for the whole curve.

Once the curve of the 1:50 plasma dilution is plotted, the prothrombin activity of the unknown plasma is read from its maximum.

In order to determine accelerator activity, the whole procedure is repeated with 1:75 plasma in a similar fashion. It is important to note the one minute values. The curve obtained by plotting thrombin units corresponding to each clotting time against activation time reaches a point where a maximum amount of prothrombin is converted to thrombin. The interval from the start of activation to maximum thrombin activity serves to calculate accelerator activity.

In order to determine antithrombin activity 1:25 dilutions of plasma are used. On account of the rapid transformation of prothrombin to thrombin in 1:25 plasma, the maximum of the curve is very rapidly reached (cf. Fig. 1). Special care has to be taken that the first one minute value as well as the 15-minute value are accurately measured. At one minute intervals further aliquots of 0.2 ml. are determined until it is clear where the maximum of the curve is situated. The clotting times are compared with a thrombin standard as described above. The corresponding thrombin activity of each sample is plotted and the 1:25 curve thus obtained (cf. Fig. 1).

Calculations for Compiling Coagulohepatograms

Accelerator Activity.—The accelerator activity of an unknown plasma is obtained by dividing the average time used to obtain maximum thrombin activity of a normal plasma in 1:75 dilution by the time used by the unknown plasma under similar conditions, and multiplying by 100. Under the conditions used in my two-stage method, but working at 37° C., maximum activity is reached between four and six minutes in 1:75 dilution of normal plasma using Difco thromboplastin.

Prothrombin Activity.—The prothrombin activity of the coagulohepatogram is calculated by comparison with a standard thrombin solution containing 1.60 t.u. per tube. This value corresponds closely to a plasma of 100% prothrombin activity in 1:50 dilution. It is also possible, as I have shown in a previous publication, to use lyophile plasma or normal plasma obtained the same day for comparison. This has the disadvantage of variations corresponding to the normal range of prothrombin activities.

$$\% \text{ prothrombin activity} = \frac{t_n}{t} \times 100$$

where t_n is the prothrombin time of a plasma of 100% activity and t the prothrombin time of the unknown.

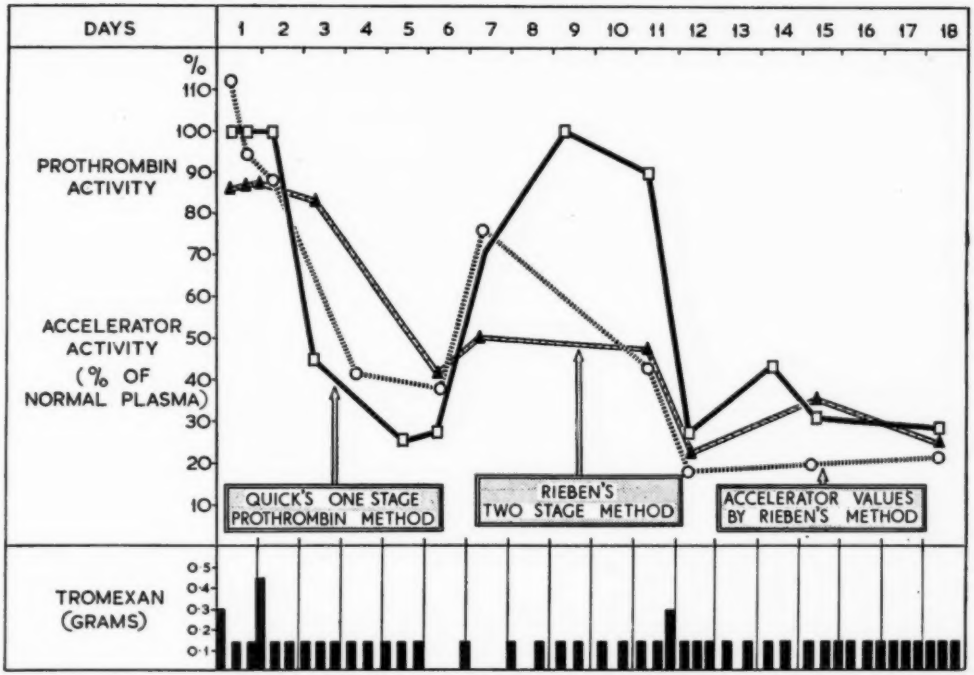

DAYS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
%
PROTHROMBIN ACTIVITY
ACCELERATOR ACTIVITY (% OF NORMAL PLASMA)
QUICK'S ONE STAGE PROTHROMBIN METHOD
RIEBEN'S TWO STAGE METHOD
ACCELERATOR VALUES BY RIEBEN'S METHOD
TROMEXAN (GRAMS)

FIG. 2

Natural Antithrombin Activity.—This can be obtained from the 1:25 curve of the coagulohepatogram (cf. Fig. 1). The difference between the highest value of the curve and the 15-minute value gives the antithrombin activity of a given tube. When highest accuracy is required, a correction can be made for the thrombin destroyed at 37° C. during the activation period of 15 minutes. For example, maximum thrombin activity of a 1:25 plasma dilution was found to be 2.61 t.u. and the 15-minute value was 1.04 t.u. In the same period the standard thrombin solution showed a decrease of activity from 1.60 t.u. to 1.25 t.u., i.e., by 0.35 units. The antithrombin effect of this tube was therefore 2.61–1.04–0.35 t.u.=1.22 antithrombin $(a.t.u.)_4$ The average normal being 1.0 a.t.u. (more accurately 0.975 ± 0.32 a.t.u. (1950a), antithrombin activity of the unknown was therefore $1.22 \times 100 = 122\%$ of average normal.

Details of interpretation of antithrombin values have been given in a previous publication (1950)). The natural antithrombin activity measured by the method presented here is quite different from heparin. Clinical evidence of the appearance of heparin in blood plasma is very rare. Significant increases of heparin have been reported in anaphylactic shock and mast cell hyperplasia (Jorpes, personal communication). The presence of heparin is not considered in the antithrombin method here described. Its effect can be quantitatively neutralized by adding 0.25 ml. of a 1:2,000 buffered solution of protamine (Hoffmann-La Roche). In the absence of heparin such an addition in no way affects the coagulohepatogram.

Normal variations of the coagulohepatogram are given in Table I in relation to its three components. The accuracy of accelerator activity determinations does not equal that of the prothrombin and antithrombin methods described in the present publication. The error of the prothrombin method is less than $\pm 2\%$.

A practical graphic presentation of the three different activities of the coagulohepatogram is obtained by representing them in three adjoining longitudinal columns; the first one corresponds to accelerator activity, the second to prothrombin activity, and the third to natural antithrombin activity. Examples of this type of graphic illustration can be found in Figs. 3, 4, 5A, and 5B. It has the great advantage of giving an excellent

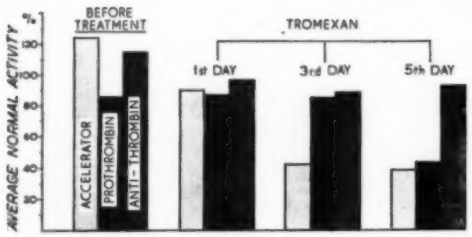


FIG. 3

visual comprehension and quantitative comparison of the three clotting factors read from a coagulohepatogram.

Applications of the Coagulohepatogram

The new concept of the coagulohepatogram was first applied to experimental studies of a dicoumarol derivative of lesser toxicity, tromexan (ethyl-ether of bis 3,3′-(4-oxycoumarinyl acetic acid). By simultaneous use of Quick's one-stage and my own two-stage procedure for prothrombin determinations, significant discrepancies of the curves were observed. The more rapid fall of values with the one-stage method (cf. Fig. 3) can be explained by the fact that following tromexan administration accelerator activity of plasma decreases. Experiments with my two-stage method have shown that results depend little on accelerator variations when working with 1 : 50 plasma dilutions. In Fig. 2 the effect of tromexan on liver function during an 18-day period is demonstrated. On the sixth and seventh day tromexan administration was stopped or greatly reduced.

As can be seen from Fig. 2, accelerator drops and returns to normal more rapidly than prothrombin measured by the two-stage technique. The practical value of coagulohepatograms is evident when one follows their changes during tromexan treatment as is illustrated in Fig. 3.

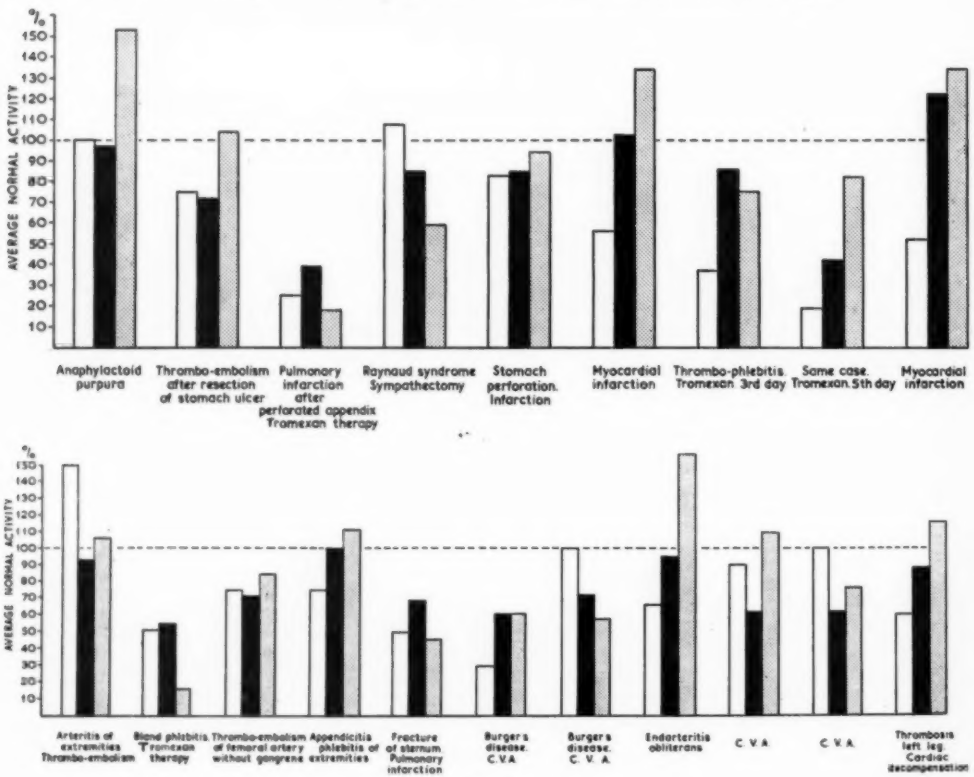


FIG. 4

The three factors of the coagulohepatogram have already undergone changes after one day of treatment. Low accelerator activity on the third day contrasts with higher values for prothrombin and antithrombin. At this moment there is a distinct dissociation of accelerator and prothrombin.

The value of the coagulohepatogram being recognized, an extended study of various normal and pathological plasmas and a screening of liver disorders and vascular diseases were undertaken. These studies will be described in detail elsewhere. Some preliminary examples are given here concerning the realm in which the coagulohepatogram seems to be of particular value, namely, in vascular and liver disorders.

Vascular Diseases.—Of 20 cases of vascular diseases and thrombo-embolic conditions studied with the new technique, the great majority showed strikingly abnormal coagulohepatograms. The findings are summarized in Fig. 4. Of particular importance are increased antithrombin values in cases of endarteritis obliterans, anaphylactoid purpura, and two representative cases of myocardial infarction.

Disorders of the Liver and Bile Ducts.—Pathological values of accelerator activity, i.e., values below 60%, were frequently observed in parenchymatous liver diseases. As could be expected, low values were found in cases treated with dicoumarol or tromexan and also in lyophile plasma. Measured with the author's two-stage procedure, the prothrombin activity of the latter is normal.

In Figs. 5A and 5B coagulohepatograms of representative cases of a variety of liver diseases and diseases of the bile ducts are given under seven different headings.

In cholelithiasis normal coagulohepatograms were obtained in a few instances. In several cases abnormal coagulohepatograms became normal following cholecystectomy. Depression of accelerator is frequently a distinct sign of secondary liver involvement in these cases, due to bad biliary flow or to congestion. Biliary obstruction and jaundice of obstructive type were frequently accompanied by high antithrombin values. This is of definite diaguostic importance. In biliary

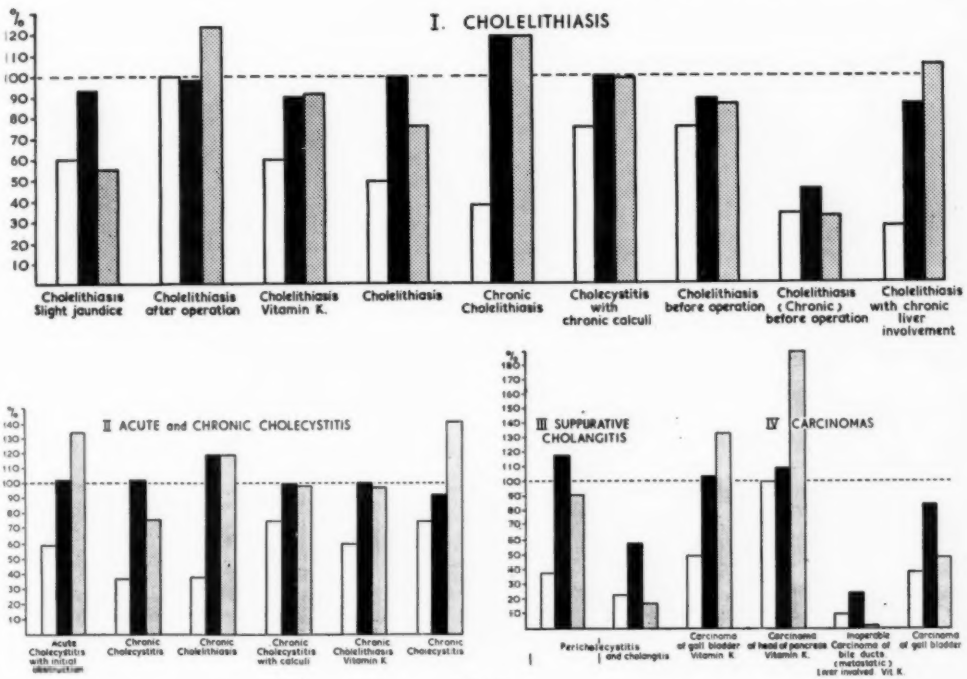


FIG. 5A

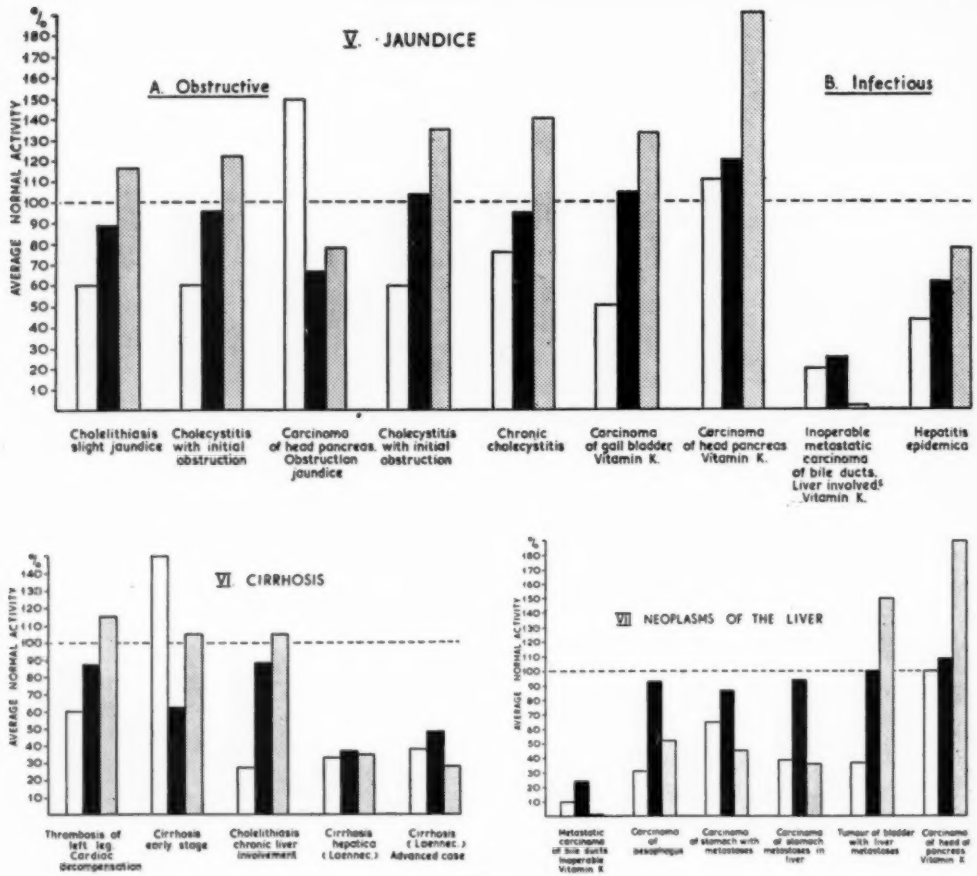

V. JAUNDICE
A. Obstructive
B. Infectious
AVERAGE NORMAL ACTIVITY
Cholelithiasis slight jaundice
Cholecystitis with initial obstruction
Carcinoma of head pancreas. Obstruction jaundice
Cholecystitis with initial obstruction
Chronic cholecystitis
Carcinoma of gall bladder Vitamin K.
Carcinoma of head pancreas Vitamin K.
Inoperable metastatic carcinoma of bile ducts. Liver involved Vitamin K.
Hepatitis epidemica
VI. CIRRHOSIS
Thrombosis of left leg. Cardiac decompensation
Cirrhosis early stage
Cholelithiasis chronic liver involvement
Cirrhosis hepatica (Laennec.)
Cirrhosis (Laennec.) Advanced case
VII. NEOPLASMS OF THE LIVER
Metastatic carcinoma of bile ducts Inoperable Vitamin K
Carcinoma of oesophagus
Carcinoma of stomach with metastases
Carcinoma of stomach metastases in liver
Tumour of bladder with liver metastases
Carcinoma of head of pancreas Vitamin K

FIG. 5B

obstruction high accelerator values have recently been reported by Owren (1949), who uses a more elaborate method. In several cases of jaundice of short duration (Fig. 5B) values of 60% were obtained.

Abnormal coagulohepatograms are important in a large proportion of cases of jaundice, and last but not least in various early and late phases of liver cirrhosis. This is borne out by Fig. 5B. The striking behaviour of the three factors involved in the coagulohepatogram in neoplastic liver diseases should also be mentioned (Fig. 5B). In early involvement of liver parenchyma, accelerator activity may be decreased. In more advanced cases of subacute and chronic hepatitis, accelerator is most often depressed. Attention was drawn to the fact that early parenchymatous liver disease and early liver damage can be revealed by the accurate two-stage prothrombin method (Rieben, 1946b). In a previous communication (1950), in confirmation of Volkert (1942), an initial fall of antithrombin was described in liver damage due to obstructive jaundice, followed in most cases by a distinct rise above normal. In neoplastic liver disease, be it primary or secondary, the degree of obstruction can often be estimated by a glimpse at the coagulohepatogram which shows a rise of antithrombin activity.

Summary and Conclusions

The coagulohepatogram is a new method of appraisal of three vital partial functions of the liver quite distinct from non-specific liver function tests. The factors surveyed by this simple, easily remembered and visually very comprehensive method show dissociations in various vascular, haemorrhagic, thrombo-embolic, and liver disorders. Experimentally, changes of the coagulohepatogram following tromexan treatment are a striking example.

The clinical significance of the coagulohepatogram is greatest in conditions where any of the three liver functions on which it is based may undergo changes. Accelerator and prothrombin activity show dissociation following tromexan and in acute and chronic liver disease. Thus the coagulohepatogram gives us insight into a most delicate triad of liver functions by permitting simultaneous appraisal of three specific liver substances known in isolated form.

Studies are now focused on a survey of various stages of acute, subacute, and chronic liver disorders, eclampsia, and diseases of the newborn. To the clinician the coagulohepatogram is a new diagnostic tool valuable in the differential diagnosis of liver diseases. It helps him to obtain a survey of three sensitive partial functions of the liver. The coagulohepatogram thus fulfils the criteria proposed for modern liver function tests, namely, specificity and quantitative appraisal.

REFERENCES

Brinkhous, K. M., Smith, H. P., and Warner, E. D. (1937). *Amer. J. med. Sci.*, **193**, 475.
Herbert, F. K. (1940). *Biochem. J.*, **34**, 1554.
Jorpes, Karolinska Institutet, Stockholm. Personal communication.
Owren, P. A. (1947). *Acta med. scand.*, Suppl. 194, p. 247.
—— (1949). *Scand. J. clin. Lab. Invest.*, **1**, 131.
Quick, A. J. (1938). *J. Amer. med. Ass.*, **110**, 1658.
—— (1945). *Amer. J. clin. Path.*, **15**, 560.
Rieben, W. (1946a). *Schweiz. med. Wschr.*, **76**, 725.
—— (1946b). *Helv. med. Acta*, **13**, 302.
—— (1950). *Sang*, **21**, 493.
—— (1952). *Schweiz. med. Wschr.* In the press.
Volkert, M. (1942). *Acta physiol. scand.*, **5**, Suppl. 15.
Ware, A., and Seegers, W. (1948). *J. biol. Chem.*, **172**, 699.
Warner, E. D., Brinkhous, K. M., and Smith, H. P. (1936). *Amer. J. Physiol.*, **114**, 667.
—— and Owen, C. A. (1942). *Amer. J. med. Sci.*, **203**, 187.
Ziffren, S. E., Owen, C. A., Warner, E. D., and Peterson, F. R. (1942). *Surg. Gynec. Obstet.*, **74**, 463.

J. clin. Path. (1952), 5, 266.

THE RELATION BETWEEN POLYAGGLUTINABILITY OF ERYTHROCYTES *IN VIVO* AND THE HÜBENER-THOMSEN-FRIEDENREICH PHENOMENON

BY

J. REEPMAKER

From the Central Laboratories of the Netherlands Red Cross Blood Transfusion Service, and the University Laboratory of Hygiene, Amsterdam

(RECEIVED FOR PUBLICATION DECEMBER 20, 1951)

Hübener (1925) and Thomsen (1927a and b, 1928) were the first to report that erythrocytes from old blood samples might sometimes be agglutinated by sera irrespective of their blood groups; in other words, they become panagglutinable. Friedenreich (1930) demonstrated that this phenomenon, which may be a cause of error in blood grouping, was due to contamination of the blood with certain enzyme-producing bacteria which cause a new agglutinogen, "T," to appear at the red cell surface and it reacts with an agglutinin "T" present in all adult sera; it is apparently absent in newborn sera. The transformation process is generally known as the Hübener-Thomsen-Friedenreich phenomenon. The T antibody is a specific cold agglutinin which is adsorbed by "changed" cells only. It exists apart from the other known specific antibodies.

Friedenreich (1930) isolated two coryneform bacteria, "M" and "J," from old blood samples. Panagglutinability was induced either by direct inoculation of blood or with culture filtrates of these bacteria. Friedenreich also found that an enzyme with a similar action was produced by some strains of Vibrio, including *Vibrio cholerae*, and also by a strain of the *Coccobacillus pierantonii*. Other bacteria were added by later investigators, e.g., some strains of actinomycetes, especially among the acid-forming groups (von Magnus, 1936), a Gram-positive coccus (Terada, 1936), a Gram-negative coccobacillus (Kossovitch and Chabaud, 1938), a corynebacterium similar to the Orla-Jensen type, the *Corynebacterium H* (*Hektoenii*) (Davidson and Toharsky, 1940, 1942), and *Cl. welchii*, *Vibrio proteus*, pneumococci of all types, and some species of streptococci and staphylococci (Chu, 1948).

Viruses of the mumps-influenza group are also capable of forming a T antigen (Burnet, McCrea, and Stone, 1946; Stone, 1947; Chu and Coombs, 1947) and, finally, the same effect results from treatment with snake venom (Briody, unpublished data), potassium periodate (Stewart, 1949; Moskowitz and Treffers, 1950), and also with trypsin under certain conditions (Rosenthal and Schwartz, 1951).

A similar phenomenon occurring *in vivo* has been less frequently observed. This is polyagglutinability of the red cells. Twelve well-documented cases have been described in the literature (Table I). In none of them was there convincing evidence that bacterial infection caused the polyagglutinability *in vivo*.

A further case, in which an organism was isolated from the urine of the patient which was able to render red blood cells panagglutinable *in vitro*, is described in this paper.

Case History

H.B., aged 25, was healthy until 1943. He then suffered from appendicitis and scarlatina. In 1946 he developed symptoms of pyelitis and was treated with sulphonamides. In the same year he was admitted to hospital at Djakarta because of amoebiasis and orchitis, probably following epidemic parotitis. By March, 1947, he had recovered. From April, 1947, onwards he has suffered from a relapsing infection of the urinary passages. This had been treated occasionally with sulphonamides, but without lasting effect. Pyelograms showed no abnormality, and cultures and guinea-pig inoculations were negative for tuberculosis.

A diagnosis of benign but persistent urinary infection, probably with prostatitis, was made. In June, 1951, the red blood cells were found to be polyagglutinable.

Serology and Urine Culture.—Till June 22, 1951, the patient's red blood cells (group A_1), containing a strong A antigen, had been regularly used as test erythrocytes for blood group determinations without giving abnormal results. On this date, however, it was found that they were agglutinated by nearly all sera of the same blood group and also by AB sera. An investigation of the exact frequency was not made at that time, but three days later the patient's corpuscles were titrated at four different temperatures against two normal A sera as well as his own serum (Table II).

TABLE I

ANALYSIS OF CASES OF POLYAGGLUTINABILITY OF RED CELLS

Investigators	Diagnosis	Sex and Age	Blood Group	Percentage of Agglutinating Sera	Optimum Temperature (°C.)	Auto-agglutination	Bacterial Infection
Levine and Katzin (1938) ..	Pneumococcal sepsis after measles	Child 4 years	O	15	20	Absent	Pneumococci type I and II
Gaffney and Sachs (1943) ..	Congenital syphilis	Male 11 years	O	47–89	4: 1/32 16: 1/16	,,	*Treponema pallidum*
	Healthy	Female	O	73	4: 1/16 16: 1/8	,,	Absent
Basil-Jones, Sanger, and Walsh (1946)	Puerperal sepsis and purulent parametritis after partus arte praematurus	Female	O	100 (45/45)	4 and 22	,,	Gram-positive rods in parametric abscesses
Boorman, Loutit, and Steabben (1946) ..	Incomplete abortion	Female 29 years	O	+100 (38/39)	22	,,	Infection present, bacteria not isolated
	Hysterectomy for parametritis	Female 37 years	O	+100 (142/143)	22	,,	Idem
Engleson and Grubb (1949)	Parametritis	Female 28 years	B	100 (7/7)	22	Present at 22° C., not at 37° C.	,,
	Sepsis with pyodermia	Child 9 years	A	100 (7/7)	4	Present at 22° C. and 37°C	,,
Henningsen (1949) ..	Healthy	Child 4 years	O	84 (adult sera) 18·4 (child's sera)	4	?	Absent
van Loghem, van der Hart, and Heier (1951) ..	Multiple tumour; metastases	Female 54 years	A	—	16	Absent	Blood culture negative. Gram-positive diplococci once isolated from sputum
Holländer (1951)	Pneumococcal meningitis	Child 9 months	O	95 infant sera negative	22	Present 4° C. 1/32 16° C. 1/16	Pneumococci type 19
Gasser and Holländer (1951)	Acute acquired haemolytic anaemia with thrombocytopenic purpura	Child 7 weeks	O	+100 (24/25)	22	Present at 37° C.	Absent
Reepmaker (195) ..	Urine infection	Male 25 years	A	—	16	Absent	α-Haemolytic streptococcus

TABLE II

AGGLUTINATION TITRES OF PATIENT'S RED CELLS WITH TWO NORMAL GROUP A SERA AND OWN SERUM AT FOUR DIFFERENT TEMPERATURES

Serum	4° C.	16° C.	22° C.	37° C.
Patient ..	—	—	—	—
A No. 11139 ..	1:4 (+)	1:2 (+)	1:2 ±	—
A No. 11141 ..	1:8 (+)	1:8 ±	1:8 ±	1:1 (+)

The strongest reactions and the highest titres were obtained at 4° C. Auto-agglutination did not take place. By June 26, 1951, the polyagglutinability had totally disappeared.

In view of the history of chronic urinary infection the patient's urine was cultured to detetmine whether there was a relationship between the infection and the abnormal agglutinability of the red blood cells.

Two different micro-organisms were isolated and cultivated in broth. Filtrates made from these cultures were added to suspensions of thrice-washed group O erythrocytes which were then kept at room temperature for about an hour. The mixture was then centrifuged and the cells again washed three times. Two drops of a 4% suspension of treated cells were then added to two drops of AB serum on a slide and the reactions read after five minutes at room temperature. Only erythrocytes treated with a filtrate derived from a Gram-positive streptococcus were agglutinated by the AB serum.

Further Investigations.—A filtrate of a four-day-old culture of the patient's streptococcus in serum broth proved to be the most useful for our experiments. The strain was dried *in vacuo* with phosphorus pentoxide in order to prevent variation through continuous re-inoculation. Dr. Skadhauge, from the Serum Institute at Copenhagen, kindly investigated the properties of this strain. It showed α-haemolysis on 5% blood agar and did not grow on 40% bile-blood agar. It did not split arginine with the formation of NH_3, nor sodium hippurate. It fermented lactose, sucrose, and trehalose, but not mannitol, salicin, aesculin, inulin, raffinose, glycerol, glycogen, or arabinose. According to Dr. Skadhauge the streptococcus belonged to a species, also isolated from urine, which was discovered by Gardborg and Borgen (1951). Dr. Friedenreich, in a personal communication, reported that this Oslo strain also caused panagglutinability of red blood cells.

Preparation of Panagglutinable T Erythrocytes.—Four drops of packed red cells were added to a mixture of equal volumes of streptococcus filtrate and normal saline and the suspension left at room temperature for half an hour. After centrifuging the red cells were washed three times in saline so as to stop the activity of the bacterial enzyme.

If they have been sufficiently transformed, they will be found to undergo agglutination within five to 10 minutes at room temperature in AB serum on a slide. If the cell filtrate mixture has been incubated for too long, the erythrocytes may undergo spontaneous agglutination in saline. It is noteworthy that Friedenreich (1930) also observed spontaneous agglutination as the result of the transformation process being carried on too far, but, contrary to our findings, this happened only when he used bacteria instead of filtrate.

Titration of T Agglutinin.—To determine the T titre, doubling dilutions of the serum were made in test-tubes (7×60 mm.). Two drops of a 4% suspension of transformed cells were added to two drops of the serum dilution. The reactions were read microscopically after one hour at 16° C. and recorded as ++, +, (+), ±, or —.

Absorption of T Agglutinin.—The T antibody was absorbed from one volume of a given serum with two volumes of transformed packed cells. After one hour at 16° C. the cells were rapidly centrifuged and the supernatant titrated against T erythrocytes, as described above.

Elution of T Agglutinin.—The T antibody was eluted from the T cells used for the absorption test. For this purpose the cells were washed twice in cold saline and centrifuged. The supernatant fluid was pipetted off and replaced by two volumes of fresh saline. The mixture was shaken and kept for ten minutes at 45° C. in a water bath. After rapid centrifugation the supernatant fluid was again titrated with T erythrocytes.

Results

As can be seen from Table III, the agglutination reactions at 16° C. were stronger than at 22° C. and 37° C. The T agglutinin can thus be classified among the specific cold agglutinins. As a working temperature, 16° C. is preferable to 4° C., since non-specific cold antibodies, not identical with T agglutinins, are active at 4° C. The amount of T agglutinin varies in different sera, titres varying from 1 in 4 to 1 in 64 (Table IV).

TABLE III

AGGLUTINATION TITRES OF STREPTOCOCCUS-TREATED GROUP O RH-NEGATIVE RED CELLS WITH GROUP AB SERUM AT FOUR DIFFERENT TEMPERATURES

Temperature	1:1	1:2	1:4	1:8	1:16	1:32
4° C.	++	++	+	+	(+)	—
16° C.	++	++	+	+	±	—
22° C.	++	+	+	(+)	±	—
37° C.	+	+	—	—	—	—

An experiment was carried out to see if red blood cells treated with the patient's streptococcus filtrate developed the same T agglutinogen as cells treated with cholera filtrate. Erythrocytes from the same normal subject were treated with cholera filtrate in a manner similar to that described for the streptococcus filtrate. One volume of packed red cells was mixed with one and a half volumes of cholera filtrate and the mixture allowed to stand for one hour. Two parallel series of serum dilutions were made; streptococcal-filtrate-treated cells were added to the first and cholera-filtrate-treated cells to the second (Table IV). The two types of cells behaved in almost the same manner.

TABLE IV

AGGLUTINATION TITRES OF STREPTOCOCCUS- AND CHOLERA-TREATED GROUP O RH-NEGATIVE RED CELLS WITH NORMAL SERA AT 16° C.

Type of Cells	Serum No.									
	5	6	7	9	14	15	17	19	26	Infant (4 months)
Streptococcus-filtrate-treated cells	1/8 (+)	1/16 ±	1/16 ±	1/32 ±	1/64 ±	1/16 ±	1/16 ±	1/4 (+)	1/4 ±	1/4 ±
Cholera-filtrate-treated cells	1/32 ±	1/16 (+)	1/16 ±	1/32 (+)	1/64 ±	1/16 ±	1/16 (+)	1/16 ±	1/8 ±	1/2 ±

It was next shown that streptococcal-filtrate-treated cells specifically adsorbed the T antibody from AB serum, with the result that this serum failed to react not only with fresh streptococcal-filtrate-treated cells, but also with cholera-filtrate-treated cells. Adsorption with cholera-filtrate-treated cells gave the same result. It was also shown that an eluate of T antibody adsorbed by streptococcus-treated cells agglutinated both types of cells and the same was true of an eluate derived from cholera-filtrate-treated cells (Table V). It is concluded, therefore, that both T agglutinogens are serologically identical.

Finally, it was found that Rh-positive (D+) erythrocytes treated with streptococcus filtrate were

TABLE V

CROSS-ABSORPTION TEST

	Serum (No. 15) Absorbed with Streptococcus-treated Red Cells	Serum (No. 15) Absorbed with Cholera-treated Red Cells
Streptococcus-treated cells ..	—	—
Cholera-treated cells	—	—

CROSS-ELUTION TEST

	1:1	1:2	1:4	1:8
Dilutions of Eluate from Streptococcus-treated Cells:				
Streptococcus-treated cells	+	+	±	—
Cholera-treated cells ..	+	(+)	±	—
Dilutions of Eluate from Cholera-treated Cells:				
Streptococcus-treated cells	+	+	±	—
Cholera-treated cells ..	+	+	—	—

agglutinated in saline by a serum containing incomplete Rh antibodies (anti-D) from which the T antibodies had been previously absorbed with T-positive Rh-negative cells (Table VI).

TABLE VI

SERUM A.N. CONTAINING INCOMPLETE ANTI-D TITRATED AGAINST STREPTOCOCC L- AND CHOLERA-TREATED GROUP O D POSITIVE CELLS IN SALINE AT 37° C.

Serum Dilution:	1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128
				Agglutination				
Streptococcus-treated group O D+ cells ..	+	+	+	(+)	±	±	±	−
Cholera-treated group O D+ cells	++	+	+	+	(+)	−	−	−
Control cells: streptococcus-treated D negative cells ..	−							

The analogous action of cholera filtrate was discovered by Pickles (1946). Chu and Coombs (1947) showed that *Vibrio proteus*, *Cl. welchii*, pneumococci, and some virus strains behaved in the same manner, and Hummel (1951) described this behaviour with a bacillus resembling Friedenreich's "M" bacillus, which he had isolated from old stored blood.

Discussion

The observations now reported support the hypothesis that polyagglutinability of erythrocytes *in vivo* and panagglutinability *in vitro* may be induced by the same causal factor. In nearly all the published reports of polyagglutinability, except for the second case of Gaffney and Sachs (1943) and the case of Henningsen (1949), a bacterial infection was demonstrated as being the possible cause. The possibility that the abnormality of the red cells and the accompanying infection are related seems especially likely in the cases with pyogenic sepsis (Basil-Jones, Sanger, and Walsh, 1946; Engleson and Grubb, 1949) and in the two pneumococcal infections (Levine and Katzin, 1938; Holländer, 1951), because Chu (1948) demonstrated that these organisms might cause panagglutinability *in vitro*. However, no attempts seem to have been made to test for the ability of the isolated micro-organism to render red cells panagglutinable. Similarly, no clear relationship was demonstrated by van Loghem, van der Hart, and Heier (1951) and Gasser and Holländer (1951). In the case of van Loghem *et al.* (1951) it is possible that enzymes with activity similar to those derived from certain bacteria were released from neoplastic tissue (Rosenthal and Schwartz, 1951). On the other hand, Gram-positive diplococci cultivated from the sputum could have been the cause of the abnormal agglutinability in this case.

Several authors showed that the erythrocytes of their patients behaved as did erythrocytes transformed *in vitro* (Gaffney and Sachs, 1943; Boorman, Loutit, and Steabben, 1946; van Loghem *et al.*, 1951; Holländer, 1951). Henningsen (1949), however, found the agglutinin adsorbed by the cells of his blood No. 38 to be not quite identical with that adsorbed by actinomyces-treated cells.

It has been pointed out that the transient nature of polyagglutinability *in vivo* compared with the stability of the T antigen and the regularity with which cells may be transformed *in vitro* is against the hypothesis that the two phenomena are identical. However, the apparent discrepancy between these two characteristics may be explained by the fact that body-temperature is unfavourable for the action of the enzyme on red cells. It is possible also that the presence of an enzyme inhibitor in human serum may weaken or prevent the reaction. It is worth noting in this connexion that Stack and Morgan (1949) found that the serum of rabbits immunized with enzymes from *Cl. welchii* developed anti-enzyme properties. Finally, the transient character of polyagglutinability can be explained by the disappearance of the responsible enzyme with the elimination of the local infection. This was confirmed in our case.

It has often been stressed that *in vivo* the phenomenon is usually that of polyagglutinability, whilst *in vitro* the cells become panagglutinable. This is, however, not true of all the published cases (Table I), and certainly not true of those described by Engleson and Grubb (1949), Holländer (1951), and Gasser and Holländer (1951), where auto-agglutination took place.

It has also been stressed that it is not possible to transmit the polyagglutinability to fresh normal blood, as can be done with blood artificially transformed with bacteria. These failures, however, do not argue against the Hübener-Thomsen-Friedenreich phenomenon manifesting itself *in vivo*, because previous experiments have shown that sterile culture filtrates are able to produce this change. The author concludes that polyagglutinability of red cells *in vivo* is in most cases probably caused by infection with certain enzyme-producing bacteria. These bacteria need not necessarily be detectable by blood culture. The occurrence of the phenomenon in apparently healthy individuals remains unexplained.

Summary

A case is described of a patient, aged 25, with a chronic, relapsing urinary infection, whose erythrocytes showed the transient phenomenon of polyagglutinability *in vivo*. A short review of the literature is given, together with a list of the microorganisms known to cause the Hübener-Thomsen-Friedenreich phenomenon *in vitro*.

An α-haemolytic streptococcus was isolated from the urine of the patient. This organism was found to be capable of rendering red cells panagglutinable. It appeared to belong to the same species that Gardborg and Borgen (1951) isolated from urine and which had similar properties.

The T agglutinogen that arises at the surface of normal red cells after treatment with a filtrate of this streptococcus was shown to be identical with the one resulting from treatment with cholera filtrate. It was demonstrated that incomplete antibodies (anti-D) could be detected in saline with Rh-positive red cells previously treated with the bacterial filtrate.

The relationship between polyagglutinability of erythrocytes taking place *in vivo* and the Hübener-Thomsen-Friedenreich phenomenon is discussed. It is concluded that these phenomena are intimately related and most probably identical.

This paper has been prepared with the technical assistance of Miss J. Willems and Miss M. van der Hart, whom I wish to thank.

I am indebted to Dr. F. Dekking for the cultivation of the bacteria and to Dr. K. Skadhauge and Dr. V. Friedenreich for investigating the properties of the streptococcus.

All the clinical data were kindly provided by Dr. P. G. Hoorweg, of the Binnengasthuis, Amsterdam.

I wish to thank Dr. J. J. van Loghem, Jr., for his kind help and valuable criticism.

REFERENCES

Basil-Jones, B., Sanger, R. A., and Walsh, R. J. (1946). *Nature, Lond.*, **157**, 802.
Boorman, K. E., Loutit, J. F., and Steabben, D. B. (1946). Ibid., **158**, 446.
Briody, B. A. Unpublished data.
Burnet, F. M., McCrea, J. F., and Stone, J. D. (1946). *Brit. J. exp. Path.*, **27**, 228.
Chu, C. M. (1948). *Nature, Lond.*, **161**, 606.
—— and Coombs, R. R. A. (1947). *Lancet*, **1**, 484.
Davidson, I., and Toharsky, B. (1940). *J. infect. Dis.*, **67**, 25.
—— —— (1942). *J. Immunol.*, **43**, 213
Engleson, G., and Grubb, R. (1949). *Amer. J. clin. Path.*, **19**, 782.
Friedenreich, V. Personal communication to the author.
—— (1930). *The Thomsen Hemagglutination Phenomenon*. Copenhagen.
Gaffney, J. C., and Sachs, H. (1943). *J. Path. Bact.*, **55**, 489.
Gardborg, O., and Borgen, L. O. (1951). *Acta path. microbiol. scand.*, **28**, 116.
Gasser, C., and Holländer, L. (1951). *Rev. Hémat.*, **6**, 316.
Henningsen, K. (1949). *Acta path. microbiol. scand.*, **26**, 339.
Holländer, L. (1951). *Helv. paediat. Acta*, **6**, 149.
Hübener, G. (1925). *Z. ImmunForsch.*, **45**, 223.
Hummel, K. (1951). Ibid., **108**, 233.
Kossovitch, N., and Chabaud, A. (1938). *C.R. Soc. Biol., Paris*, **128**, 851.
Levine, P., and Katzin, E. M. (1938). *Proc. Soc. exp. Biol., N.Y.*, **39**, 167.
Loghem, J. J. van, Hart, M. van der, and Heier, A. M. (1951). *Bull. centr. Lab. Bloedtr. Ned. Rode Kruis.*, No. 2, 93.
Magnus, R. von (1936). Thesis, *Undersögelser over en Gruppe Actinomyceter isolerede fra menneskets Svaelg* (with English summary). Copenhagen.
Moskowitz, M., and Treffers, H. P. (1950). *Science*, **111**, 717.
Pickles, M. M. (1946). *Nature, Lond.*, **158**, 880.
Rosenthal, M. C., and Schwartz, L. I. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 635.
Stack, M. V., and Morgan, W. T. J. (1949). *Brit. J. exp. Path.*, **30**, 470.
Stewart, F. S. (1949). *J. Path. Bact.*, **61**, 456.
Stone, J. D. (1947). *Aust. J. exp. Biol. med. Sci.*, **25**, 137.
Terada, S. (1936). Thesis, *J. med. Ass. Formosa*, **35**, 1285. Cited by Davidson, I., and Toharsky, B. (1940).
Thomsen, O. (1927a). *Z. ImmunForsch.*, **52**, 85.
—— (1927b). *C.R. Soc. Biol., Paris*, **96**, 556.
—— (1928). *Z. ImmunForsch.*, **57**, 301.

J. clin. Path. (1952), **5**, 271.

DETERMINATION OF THE UNSATURATED IRON-BINDING CAPACITY OF SERUM

BY

S. VENTURA

From the Postgraduate Medical School of London

(RECEIVED FOR PUBLICATION SEPTEMBER 28, 1951)

This paper deals with a modification of the methods suggested by Rath and Finch (1949) and Cartwright and Wintrobe (1949) for the determination of the unsaturated iron-binding capacity of the serum.

Transport of iron by the blood plasma may be effected through union with a β_1-globulin contained in the IV-7 fraction of Cohn (1945); this fraction has been called siderophyllin (Schade and Caroline, 1946; Laurell, 1947; Surgenor, Koechlin, and Strong, 1949). Various methods of determination indicate that the amount of siderophyllin present in 100 ml. of normal plasma is sufficient to bind about 300 μg. of iron. Only about one-third of this protein is normally saturated with iron in the plasma or serum, the metal constituting the serum iron. The sum of the iron and the further amount of iron required to saturate all siderophyllin (unsaturated iron-binding capacity or U.I.B.C.) gives a measure of the total iron-binding capacity of the serum.

Monasterio and Lattanzi (1943) and Waldenström (1944) measured the U.I.B.C. by the difference between serum iron levels before and after intravenous injection of an ionizable form of iron. Schade and Caroline (1946) employed a microbiological method based on the fact that *Shigella dysenteriae* grows only in the presence of ionized iron, and these authors noted the change in colour of the iron-binding protein when linked with the metal. Rath and Finch (1949) and Cartwright and Wintrobe (1949) estimated siderophyllin on the basis that the intensity of the red produced by union with iron is directly proportional to the amounts of the two components.

Premises for a Simplified Estimation of Unsaturated Iron-binding Capacity

The addition of sufficient ionic iron to a sample of serum to saturate all the siderophyllin present results in the appearance of a red colour, the intensity of which is directly proportional to the amount of the iron-binding protein; the quantity of iron thus bound gives the most convenient measure of the amount of the protein.

The red compound formed in serum by adding ionized iron has E max 460 mμ, and is dissociated at *p*H values on the acid side of neutrality (Figs. 1 and 2). This behaviour parallels the known properties of crystalline siderophyllin combined with iron *in vitro*. The $E\frac{1\%}{1\text{ cm.}}$ for the pure substance was determined by Surgenor *et al.* (1949), but cannot be applied to serum, as interference may occur from the presence of such substances as bile pigments and haemoglobin (Rath and Finch, 1949; Cartwright and Wintrobe, 1949).

In the present work the change in optical density of normal and pathological sera has been studied after the addition of fixed increments of ionized iron. The procedure is carried out in the cell of

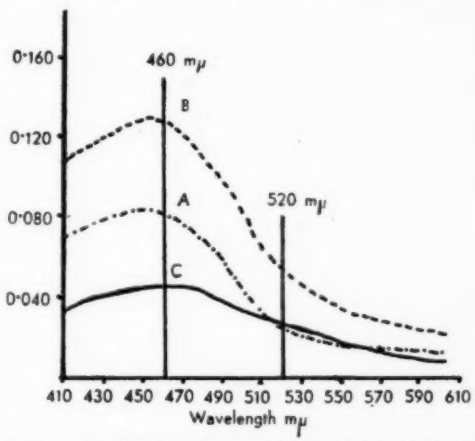


FIG. 1.—Absorption curve of serum before and after the addition of iron to saturate the siderophyllin fraction. A = serum plus saline. B = serum plus added iron. C = difference between A and B, representing absorption of iron-bound to protein.

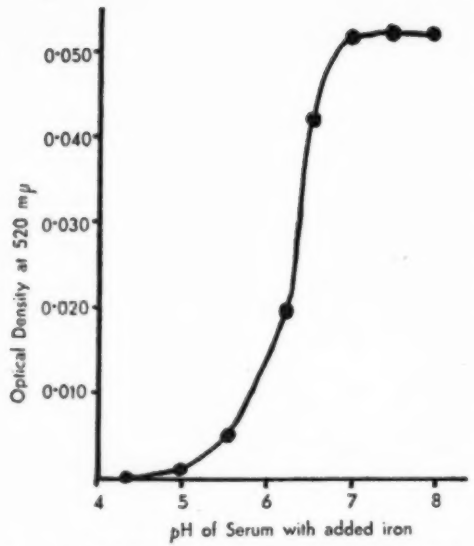


FIG. 2.—Optical density of serum at 520 mμ after the addition of iron at various hydrogen ion concentrations.

the Beckman spectrophotometer, and optimal conditions have been defined for the dilution of serum and the wavelength at which an increase in density is measured.

Dilution of the serum (1 ml.) is best made with 1.5 volumes of 0.85% sodium chloride solution. Veronal buffer is avoided, since the veronal itself combines with iron.

Fe^{++} is bound more rapidly and the reaction is more reproducible than with Fe^{+++}, as judged by the increase in optical density with time (Fig. 3).

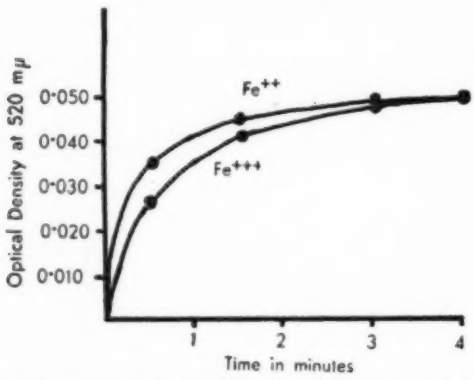


FIG. 3.—The rate of reaction of added iron in the ferrous and ferric states with the iron-binding protein of sera.

The optimum wavelength for measuring the increase in density has been found to be 520 mμ, as measurements at this wavelength are free from the influence of interfering substances observed at the maximum 460 mμ. The readings are made against a 0.85% sodium chloride solution blank.

Serum is separated at 37° C. from clotted venous blood samples. Estimations are carried out within 12 hours of taking the blood, or low values may be observed. No advantage has been found from Laurell's (1947) suggestion of first freezing the serum at −20° C. for 24 hours.

Construction of a Standard Calibration Curve

One hundred and fifty samples of sera from normal and anaemic individuals, and from normal and anaemic pregnant women, have been used. From 1 to 5 μg. of Fe^{++}, in increments of 1 μg., were added to samples of 1 ml., the increase in optical density at 520 mμ being recorded. Normal sera showed no further increase in density with the addition of Fe^{++} above 3 μg., but, with 15 sera from anaemic patients, saturation had not occurred after the addition of 5 μg. (1 μg. of Fe^{++} added to 1 ml. of serum is equivalent to the saturation of an unsaturated iron-binding capacity of 100 μg./100 ml.) For any observed increment in density △D, the U.I.B.C. is the product of △D and a factor K, derived from the average of the quotient of added iron in μg. over the determined average densities in the above experimental series (Table I).

TABLE I

DATA FOR THE CALIBRATION CURVE FOR DETERMINATION OF THE UNSATURATED IRON-BINDING CAPACITY OF SERUM

Quantity of Fe^{++} added to 1 ml. Serum	Average Increase of Optical Density at 520 = D	Coefficient of Variation (%)	$K = \frac{\mu g \text{ of added } Fe^{++}}{D}$	No. of Sera Examined	Percentage of Total
1 μg.	0·0106	6·26	94·4	150	100
2 μg.	0·0221	3·73	90·7	141	94
3 μg.	0·0329	2·91	91·3	105	70
4 μg.	0·0437	2·50	91·8	30	20
5 μg.	0·0549	1·17	91·3	15	10
			Mean value of K = 91·9		

The average values for densities after each increment of iron are shown in Table I. In Fig. 4 the average increments in optical density of the

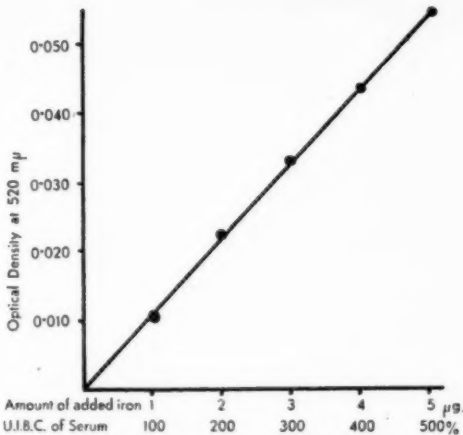


FIG. 4.—Standard calibration curve for determination of the unsaturated iron-binding capacity of serum, based on the average increases in optical density after the addition of fixed increments of ferrous iron to 150 samples of serum.

serum at 520 mμ are plotted against the corresponding amount of added iron. The points lie in a straight line. The $E\frac{1\%}{1\,cm.}$ of serum siderophyllin may be derived, knowing that 0.4 μg. of iron saturate 0.321 mg. of the protein (Surgenor *et al.*, 1949). The average increase in density of 1 ml. of serum diluted to 2.5 ml. on the addition of 1 μg. of iron is approximately 0.011 at 520 mμ and 0.019 at 460 mμ; this increase in density corresponds to the addition of 0.4 μg. of iron to 1 ml. of diluted serum or to its binding by 0.321 mg. of siderophyllin. Hence 1 g./100 ml. of serum siderophyllin saturated with iron will have a density of $\frac{0.019 \times 1000}{0.321 \times 100} = 0.59$ at 460 mμ. This is in good agreement with the value of 0.57 obtained by Surgenor *et al.* (1949) for the crystalline protein saturated with iron.

Technique

Standard Iron Solution.—This contains 70 μg. of Fe^{++} per ml. To make the solution, 122.5 mg. of Analar ferrous ammonium sulphate are dissolved in 5 ml. of N-acetic acid, and the volume made up to 250 ml. with double-distilled water (0.1 ml. of the solution added to 1 ml. of serum does not change the *p*H of the latter).

Procedure.—First 1 ml. of serum is measured directly into the Beckman spectrophotometer cell and diluted with 1.5 ml. of 0.85% sodium chloride solution in double-distilled water. The blank cell is filled with 2.5 ml. of the saline solution alone. The density of the test cell is then read against the blank at 520 mμ, and 0.1 ml. of the standard iron solution added to the test, mixed with a glass rod for two minutes, and the increase in density observed after standing for a further four minutes. The added iron is 7 μg., which is sufficient to saturate an unsaturated iron-binding capacity of 700 μg. per 100 ml. of serum, that is, sufficient excess of iron is added to satisfy any unsaturated iron-binding capacity likely to be encountered.

The observed increase in density (△D) is converted to unsaturated iron-binding capacity expressed as μg. per 100 ml. of serum, by multiplying by K, the value of which was 91.9 from the calibration curve given in Fig. 4, and by 100.

All glassware and cells are washed with iron-free distilled water.

Comparison of the Present Procedure with Other Techniques

To establish the accuracy of the suggested procedure, the unsaturated iron-binding capacities of 10 different sera were determined according to four different methods. The results are given in Table II.

From Table II and Fig. 5 it may be seen that there is good agreement with Cartwright and Wintrobe's method. The advantage of the present procedure lies in its speedier mode of execution. Laurell's method, and that based on the intravenous injection of an easily ionized iron compound, sometimes give lower results. In one sample of serum, 10 determinations gave a coefficient of variation of 2.74%. It should be noted that, although the absolute increases in optical density are of the order of only 1 to 5% when

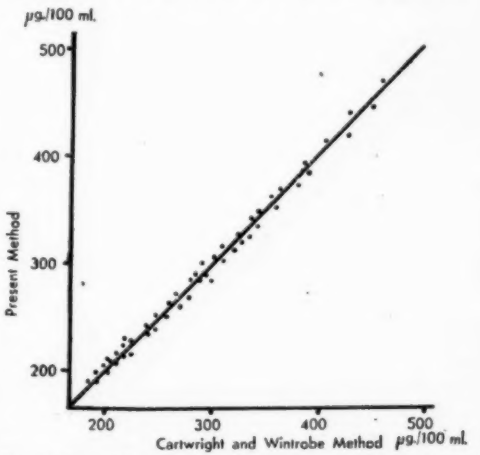


FIG. 5.—Scatter diagram comparing the values for the unsaturated iron-binding capacity of 50 serum samples determined by the method of Cartwright and Wintrobe (1949) and by the present method.

TABLE II

COMPARISON OF RESULTS OF DETERMINATION OF UNSATURATED IRON-BINDING CAPACITY OF SERUM BY VARIOUS METHODS

Case No.	Methods			
	Present Method	Cartwright and Wintrobe (1949)	Laurell (1947)	Intravenous Injections of Fe
1	230	217	206	198
2	470	458	405	417
3	371	364	360	352
4	216	225	214	—
5	229	225	240	201
6	326	337	305	299
7	317	309	311	—
8	269	278	239	247
9	198	205	200	—
10	325	319	296	305

the unsaturated iron-binding capacity of sera containing from 100 to 500 μg. per 100 ml. are saturated with iron, the readings are carried out with the same solution and cell, with consequent minimization of attendant experimental errors.

Summary

A method for determining the unsaturated iron-binding capacity of a 1 ml. sample of serum has been described. The procedure is carried out in the Beckman spectrophotometer.

The accuracy of the method compares favourably with other available methods, and has the advantages of speed and simplicity.

I am indebted to Professor E. J. King and to Drs. I. Wootton and J. C. White for interest and advice in the preparation of this paper.

REFERENCES

Cartwright, G. E., and Wintrobe, M. M. (1949). *J. clin. Invest.*, **28**, 86.

Cohn, E. J. (1945). *Science*, **101**, 51.

Laurell, C. B. (1947). *Acta physiol. scand.*, **14**, Suppl. 46.

Monasterio, G., and Lattanzi, A. (1943). *Rass. Fisiopat. clin. ter.*, **15**, 141.

Rath, C. E., and Finch, C. A. (1949). *J. clin. Invest.*, **28**, 79.

Schade, A. L., and Caroline, L. (1946). *Science*, **104**, 340.

Surgenor, D. M., Koechlin, B. A., and Strong, L. E. (1949). *J. clin. Invest.* **28**, 73.

Waldenström, J. (1944). Quoted by Cartwright and Wintrobe (1949).

J. clin. Path. (1952), **5**, 275.

THE APPLICATION OF A HAEMAGGLUTINATION TECHNIQUE TO THE STUDY OF *TRICHOMONAS VAGINALIS* INFECTIONS

BY

M. G. McENTEGART

From the Department of Bacteriology, University of Liverpool

(RECEIVED FOR PUBLICATION JANUARY 16, 1952)

Although there is an extensive literature on the subject of trichomoniasis, reports on the serological investigation of *Trichomonas vaginalis* infections are scanty and the results inconclusive, the emphasis in serological work having been on bovine infections with *Trichomonas foetus* rather than on human infections with *T. vaginalis*.

Riedmüller in 1932 (quoted by Trussell, 1947) tested the sera of a number of women for complement-fixing antibody using *T. foetus* as antigen. His results were uniformly negative irrespective of the presence or absence of trichomonas infection. Wendlberger (1936) carried out complement fixation tests with an alcoholic extract of a culture of *T. vaginalis* as antigen. The culture employed for the production of this antigen contained bacterial contaminants. Positive results were observed in 22 out of 32 sera taken from known cases of trichomoniasis, while 16 sera from apparently healthy women were negative. A series of 400 sera was examined by Trussell, Wilson, Longwell, and Laughlin (1942) by a complement fixation technique with a washed suspension of *T. vaginalis* as antigen. The test was positive with 52 out of 110 sera from known cases of trichomoniasis (47%). Of 290 "normal" female sera tested, 48 were positive (16%).

The results recorded in the latter two papers provide the only evidence for the occurrence of serum antibodies in human trichomoniasis at present known to the author.

A micro-agglutination technique was introduced in 1941 by Robertson working with *T. foetus*. This test was shown to give valuable information in the investigation of *T. foetus* infections of cattle (Kerr and Robertson, 1941; Pierce, 1947). Trussell (1946) applied a similar technique to human infections with *T. vaginalis*. Having established that the method would give successful results in experimentally immunized rabbits, 200 human sera were examined, but over 90% of these were recorded as negative and the remainder gave only low titre positive reactions.

Both complement fixation and micro-agglutination methods for the detection of antibody to *T. vaginalis* were tested in this laboratory, but they gave only weak reactions with human serum.

A new approach to the problem was suggested by the observations of Muniz (1950), who used a haemagglutination technique similar to that employed by Middlebrook and Dubos (1948) in their studies on tubercle bacilli. He applied it to investigations on trypanosomiasis, sensitizing erythrocytes with a polysaccharide fraction of *Schizotrypanum cruzi*, and observing lysis rather than agglutination as evidence of positive reactions. An attempt was therefore made to apply the technique of agglutination of sensitized red cells for the detection of circulating antibodies in human, trichomoniasis. The results are recorded below.

Methods

Cultivation of *T. vaginalis*.—The isolation of *T. vaginalis* and its cultivation in laboratory media without bacterial contaminants was formerly a difficult problem. Once antibiotics became available it was soon discovered that the addition of these substances to the medium allowed the protozoa to grow while suppressing the bacterial contaminants. Unfortunately the only media which would give an adequate growth of protozoa contained agar or some other solid constituent which made difficult the harvesting of the organisms after they had grown. This was overcome and satisfactory growth obtained in the absence of the solid component by incubating the cultures anaerobically.

The medium which was found to be most suitable for routine use was a modification of the cysteine-peptone-liver-maltose (C.P.L.M.) media described by Johnson and Trussell (1943), the agar being omitted and "difco" bacto-tryptose substituted for bacto-peptone; the methylene blue indicator was also omitted. Thus the final composition of the medium was as follows:

Bacto-tryptose	3·2 g.
Cysteine hydrochloride	0·24 g.
Maltose	0·16 g.
Ringer solution	96·0 ml.
Difco liver infusion	32·0 ml.

The Ringer solution for the C.P.L.M. medium was composed as follows:

NaCl	0·6%
$NaHCO_3$	0·01%
KCl	0·01%
$CaCl_2$	0·01%

No *p*H adjustment was necessary. As heat-sterilized batches were less successful than those which had been Seitz-filtered, the latter method of sterilization was adopted throughout the investigation. The original C.P.L.M. medium contained 10% of human serum, but as it was hoped to immunize rabbits with the cultures the same percentage of rabbit serum was substituted for human serum. The serum was inactivated at 56° C. for 30 minutes and added immediately before the medium was to be inoculated.

This medium does not keep well, and must be used within about a week of preparation.

Isolation of Protozoa from Vaginal Swabs.—Strains of *T. vaginalis* employed in this investigation were isolated from vaginal swabs taken from patients reporting to a local gynaecological out-patient department. Specimens were only taken for culture when direct examination of the vaginal discharge showed numerous active flagellates. Antibiotics were added to the freshly prepared medium to give the following final concentrations:

Penicillin and streptomycin	1,000 u./ml.
Chloromycetin	500 „

The vaginal swabs taken from patients were placed in a tube containing about 1 ml. of the above medium. They were then sent directly to the laboratory, or, if this was not convenient, could be incubated at 37° C. overnight without ill effect. On arrival in the laboratory the culture medium was examined for active trichomonads and subcultured to 10 ml. volumes of the same medium; these tubes of media were then incubated anaerobically. Cultures were examined for active flagellates every two to three days and subcultured to fresh medium. After a few passages it was generally possible to obtain a bacteria-free culture of the protozoa. The absence of bacterial contaminants was as a rule evident from the macroscopic appearance of the culture, the protozoa after three days' incubation giving a fine granularity to the medium quite unlike the turbidity produced when bacteria were present.

When a new strain was isolated in "pure" culture, subcultures were incubated aerobically and anaerobically to establish that it was in fact free from bacterial contaminants. It was found that if antibiotics were now omitted from the culture medium, poor growth was obtained, and this seemed to be mainly the result of the absence of streptomycin; consequently, antibiotics were incorporated in the medium throughout the investigation.

Preparation of Immune Rabbit Serum.—The trichomonads from 100 ml. of a four-day culture in modified C.P.L.M. medium were deposited by centrifugation and washed three times in saline before being re-suspended in 10 ml. of saline. The average number of organisms now present was estimated by counting in a haemocytometer and found to be approximately 10^7 per ml. This suspension was used to immunize a rabbit which had previously been bled to obtain a control sample of serum. Two injections were given intravenously each week for three weeks, beginning with 0.5 ml. in the first week, 1.0 ml. in the second week, and 1.5 ml. in the third week. Four days after the final injection the rabbit was bled and the serum separated.

As a preliminary test both pre- and post-immunization sera were tested for complement-fixing antibodies using as antigen the suspension of trichomonads with which the rabbit had been immunized. This test demonstrated the presence of some antibody in the immune serum, though the titre (1/10) was very much lower than that obtained by Trussell in 1946.

Preparation of Antigen and Sensitization of Erythrocytes.—In order to obtain an extract of the trichomonads suitable for sensitizing red cells the standard laboratory methods employed in the grouping of streptococci were tried.

The deposited protozoa from 30 ml. volumes of a three-day culture were washed three times in saline and treated according to the methods described by Lancefield (1933), Fuller (1938), and Maxted (1948). The resulting extracts in a volume of 1.0 ml. were then tested undiluted for their ability to sensitize sheep erythrocytes as demonstrated by the subsequent agglutination of the treated cells by immune rabbit serum.

The attempt to destroy the enzyme in the antigen extract prepared by Maxted's method was unsuccessful, as the erythrocytes were lysed. Serial dilutions of this antigen were tested, and although lysis was eliminated by the dilution of the extract beyond 1/20 no agglutination of treated red cells could be observed at any dilution. The extract prepared by the formamide method (Fuller, 1938) gave the most promising results, agglutination of treated red cells being observed up to a dilution of 1/100 of the immune rabbit serum. There was no agglutination of sensitized cells with the control sample of serum from the same rabbit, or of untreated erythrocytes with the immune rabbit serum.

Having established that the antigen prepared by formamide extraction of the trichomonads would sensitize red cells, titrations were made to determine the highest dilution of the antigen which would produce sensitization.

In the preliminary test against immune rabbit serum it was found that 1/50 was the highest dilution of antigen capable of producing sensitization when mixed with an equal volume of a 2% suspension of washed sheep cells. In all subsequent experiments the sensitizing capacity of each antigen was titrated against the immune rabbit serum before being used to sensitize cells for the testing of other sera. Equal parts of antigen dilutions were mixed with 2% sheep red cell suspension and held for one hour at 37° C.; the mixtures were then tested with the immune rabbit serum. The highest antigen dilution, which still produced maximum agglutination, as determined by the titration of the immune rabbit serum, was taken as the optimum and used to sensitize cells in subsequent tests. A typical titration of antigen in this way is shown in Table I.

TABLE I

PRELIMINARY TITRATION OF ANTIGEN AGAINST IMMUNE RABBIT SERUM

Antigen Dilution	Immune Rabbit Serum Dilutions						Controls
	1/5	1/10	1/25	1/50	1/100	1/250	
1/5	++++	++++	++++	++++	++	−	−
1/10	++++	++++	++++	++++	++	−	−
1/25	++++	++++	++++	++++	∓	−	−
1/50	++++	++++	+++	±	−	−	−
1/100	++++	+++	+	−	−	−	−
Normal cells	−	−	−	−	−	−	−

++++ = maximum agglutination; − = no agglutination.

When human sera were tested against unsensitized sheep cells it was found that the majority showed the presence of agglutinins to low titre. In order to avoid having to absorb the sera to remove these heterophile agglutinins before testing with sensitized cells, human group O cells were tested in the hope that they could be used in place of sheep cells. This was unsuccessful. Similar attempts to sensitize rabbit erythrocytes or suspensions of bacterial cells were equally unsuccessful. It was decided, therefore, to continue using sheep cells and to absorb all sera before test with cells from the same sheep. The method of absorption is described later.

Six absorbed human sera from known cases of trichomoniasis and an equal number of control normal sera were tested for their ability to agglutinate sensitized sheep cells. Three of the sera from cases gave a positive result to a dilution of 1/100; the control sera were negative. The test was repeated with the same sera, but this time guinea-pig complement was added before placing the test in the water bath. It seemed possible that the results would be more easily read and perhaps the test made more sensitive if lysis rather than agglutination was taken as the evidence of the antibody–antigen reaction. Lysis occurred in the presence of complement to the same dilution as agglutination had occurred in its absence. As there was no striking increase in sensitivity it was decided to continue the investigation, taking the occurrence of agglutination as evidence of the presence of serum antibody. Once the serum had been absorbed it was possible to obtain a quick qualitative result by slide agglutination, but this gave less information than did a full test with a series of dilutions of the absorbed serum in small tubes.

There was no suggestion in titrations such as that shown in Table I that an excess of free antigen might inhibit the agglutinating action of the serum on sensitized cells. None the less, it was felt that the possibility of such an inhibitory effect had to be carefully excluded before using the technique in the examination of any extended series of human sera.

Five rows of tubes were set out, each row containing the same series of dilutions of immune rabbit serum. An equal volume of an antigen dilution was added to each row. Thus to each tube in the first row was added 1/5 antigen, to each in the second row 1/10, and so on for 1/25 and 1/50 dilutions of the antigen; the fifth row served as a control and to this an equal volume of normal saline was added. These serum-antigen mixtures were placed in the water bath at 37° C. for 30 minutes and then tested in the usual way for their ability to agglutinate sensitized cells. There was a slight reduction in the agglutinating titre in the presence of antigen dilutions 1/5 and 1/10, the end-point being about one serum dilution lower than in the control series (Table II). It was, however, unlikely that any excess of antigen in the cell suspensions employed would significantly reduce the agglutinating titre of the serum under test.

In order to test this under working conditions one further experiment was performed. A 2% suspension of sheep erythrocytes was mixed with an equal volume of a 1/10 dilution of antigen and incubated at 37° C. for one hour. After incubation, half of the suspension was removed and centrifuged, the deposited cells washed and re-suspended in saline to the original volume. This suspension of washed sensitized cells and the original suspension were then tested against immune rabbit serum. There was only a slight difference observed in the degree of agglutination at the end-point, the same end-point being obtained with the two suspensions. It was thus concluded that it would be reasonable to omit the washing and re-suspension of the red cells to be used in routine agglutination tests.

TABLE II

AGGLUTINATION OF SENSITIZED ERYTHROCYTES BY IMMUNE RABBIT SERUM IN EXCESS ANTIGEN

Dilution of Antigen	Immune Serum Dilutions					Control Saline Only
	1/5	1/10	1/25	1/50	1/100	
1/5	++++	++++	++	++	−	−
1/10	++++	++++	++	++	±	−
1/25	++++	++++	+++	++	++	−
1/50	++++	++++	+++	++	++	−
Control, no antigen ..	++++	++++	++++	+++	++	−

++++ = maximal agglutination; − = no agglutination.

Technique for Testing Human Sera.—The following method was employed in all routine tests.

Preparation of Sensitized Cell Suspension.—A suitable volume of a 2% suspension of sheep cells was prepared and half of this added to an equal volume of normal saline to provide a 1% control suspension. The other half was added to an equal volume of a suitable dilution of antigen in saline (usually 1/10) and thoroughly mixed. Both suspensions of cells were then placed in the 37° C. incubator for one hour.

Absorption of Sera.—Serum for the test was diluted 1/5 (0.5 ml. to 2.0 ml. of saline) and inactivated at 56° C. for 30 minutes. After inactivation 0.1 ml. of packed sheep cells was added to the serum and allowed to act for 20 minutes in the 37° C. incubator; the tubes containing the serum red cell mixture were then centrifuged to deposit the cells and the procedure repeated without removing the supernatant from the deposited cells. After the second period of absorption the samples were again centrifuged, the supernatant serum removed, and serial dilutions prepared for testing.

Performance of the Test.—Serial dilutions of the adsorbed serum were distributed in 0.2 ml. volumes in 3 in. × ½ in. tubes. The first three dilutions were duplicated for testing with normal sheep cells in order to exclude heterophile agglutination. Then 0.2 ml. of sensitized red cells was added to each tube in the series and 0.2 ml. of normal cells to the three control dilutions. Two further controls were included, each containing 0.2 ml. of saline to exclude non-specific agglutination of the two cell suspensions. The contents of the tubes were mixed and the whole test placed in the water bath at 37° C. for 30 minutes.

Although gross agglutination could be detected immediately after the tubes were removed from the water bath, it was necessary to allow the red cells to sediment, completely before the results were finally read. Reading of results was based on the appearance of the plaque of red cells appearing at the bottom of the tube and checked by gentle agitation to re-suspend the agglutinated cells.

An arbitrary system of noting the results was employed, the agglutination being recorded as ++++, +++, etc., according to the degree observed. The agglutinating titre was taken as the last tube showing at least +++ agglutination.

Results

Altogether 10 " strains " of *T. vaginalis* have been isolated from patients, but only six of these have been used for the preparation of antigen. Table III, abstracted from the individual titrations, shows the type of results obtained.

Four of these six antigens were tested against two bovine sera known to contain antibody against the serologically distinct Manley and Belfast strains of *T. foetus* and against the standard vaginalis rabbit serum. The results obtained are shown in Table IV.

It will be seen that agglutination of red cells sensitized with *T. vaginalis* extract is produced by *T. foetus* antisera with all antigens tested, but that the titre in each case is lower than those obtained with the immune rabbit serum. The converse reaction, i.e., testing *T. vaginalis* immune sera against cells sensitized with *T. foetus* extracts, has not so far been investigated.

One hundred samples of " normal " human serum were obtained from the Regional Transfusion

TABLE III

COMPARATIVE TITRES OF SIX ANTIGENS TESTED AGAINST IMMUNE RABBIT SERUM

Antigen 1/10	Immune Rabbit Serum Dilutions						
	1/5	1/10	1/25	1/50	1/100	1/250	1/500
Dodd ..	++++	++++	++++	++++	++++	–	–
Dean ..	++++	++++	+++	+++	++	–	–
Gibson	++++	++++	+++	++	±	–	–
Beardmore	++++	++++	+++	+++	+	±	–
Griffith..	++++	++++	++	+	±	–	–
Macieson	++++	++++	++++	++++	++	+	–

++++ = maximum agglutination; – = no agglutination.

TABLE IV

ANTIGENS PREPARED FROM DIFFERENT " STRAINS " OF *T. vaginalis* TESTED AGAINST IMMUNE BOVINE SERA AND IMMUNE RABBIT SERUM

Antigen	Serum*	Serum Dilutions						Saline Control
		1/5	1/10	1/25	1/50	1/100	1/250	
Gibson ..	Manley	++++	++++	++	–	–	–	–
	Belfast	++++	++++	+	–	–	–	–
	Rabbit vaginalis	++++	++++	+++	++	±	–	
Beardmore ..	Manley	++++	++++	+	–	–	–	–
	Belfast	++++	++++	–	–	–	–	–
	Rabbit vaginalis	++++	++++	++++	+++	+	∓	–
Griffith ..	Manley	++++	++++	±	–	–	–	
	Belfast	+++	–	–	–	–	–	–
	Rabbit vaginalis	++++	++++	++	+	∓	–	
Macieson ..	Manley	++++	++++	++	–	–	–	
	Belfast	++++	++++	–	–	–	–	–
	Rabbit vaginalis	++++	++++	++++	++++	++	+	
Normal cells ..	Manley	–	–	–				–
	Belfast	–	–	–				
	Rabbit vaginalis	–	–	–				

* All sera adsorbed with sheep cells before testing. ++++ = maximum agglutination; – = no agglutination.

TABLE V

RESULTS OF HAEMAGGLUTINATION TESTS

Serum	Total No.	Negative	Positive at Dilutions							Positive		Negative	
			1/5	1/10	1/20	1/40	1/80	1/160	1/250	No.	%	No.	%
Normal male ..	50	31	6	10	3	0	0	0	0	19	38	31	62
.. female ..	50	39	3	5	3	0	0	0	0	11	22	39	78
Trichomonas vaginalis in female discharge	50	1	2	5	15	12	10	2	3	49	98	1	2
Normal children (7 male, 6 female) ..	13	6	4	3	0	0	0	0	0	7	—	6	—

Service, 50 from males and 50 from females. These were absorbed with normal sheep cells and tested for the presence of agglutinin for sensitized cells in the usual way. The results are shown in Table V; also included in this table are the results of the examination of 50 sera from women attending the V.D. Department who were selected for the purpose of the investigation because trichomonads were present in the vaginal discharge with or without producing clinical evidence of vaginitis; the table also shows the results on a small number of sera from children of both sexes below the age of puberty.

The results set out in Table V make it difficult to decide what titre should be regarded as significant. As all the sera have been absorbed and subsequently shown to be unable to agglutinate normal sheep red cells, it seems unlikely that positive results were due to the presence of heterophile antibody. If only the positive results in serum dilutions above 1/10 are considered as significant the percentage of positives in the sera from the different groups of persons is as shown in Table VI.

TABLE VI

AGGLUTINATION BY HUMAN SERA OF SHEEP RED CELLS TREATED WITH EXTRACTS OF *Trichomonas vaginalis*

Human Sera	No. Examined	Positive (%)	Negative (%)
Trichomonas vaginalis in female discharge	50	84	16
" Normal " female blood donors	50	6	94
" Normal " male blood donors	50	6	94
Children (7 males, 6 females) ..	13	0	100

Titre: 1/10 recorded as positive.

Comment

Very little is at present known about the occurrence of serum antibodies in patients with *T. vaginalis* infections.

Various serological methods have been employed, but the number of investigations is small and the results conflicting. An agglutination reaction is observed when human sera are tested against sheep cells treated with a formamide extract of *T. vaginalis*. The significance of this reaction is not easy to assess. Tokura (1935) observed that the three trichomonads which may occur in human beings, i.e., *T. hominis*, *T. buccalis*, and *T. vaginalis*, gave serologically distinct reactions when tested against immune rabbit serum, but this observation is unconfirmed; however, there remains the possibility that some of the low titre positive results recorded in Table V above may have been due to antibody to one of these strains other than vaginalis, particularly as some low titre positive reactions were obtained when bovine sera containing antibodies against *T. foetus* were tested against cells sensitized with *T. vaginalis* antigen.

Although the occurrence of positive results at low titre with the sera of children suggests some non-specific effect, it does not follow that all low titre positives are non-specific. Indeed, it is quite possible that some of the positive results excluded from Table VI are, in fact, weak specific positives. This is borne out by the low incidence of positives in normal women shown in this table, where the recorded 6% is a good deal lower than would be expected considering the general clinical opinion as to the number of women who are symptom-free carriers of *T. vaginalis*. This has recently been put as high as 24%. If the number of positives recorded in normal women is an underestimate, then it is possible that the same criticism can be applied to the results recorded for " normal " males. While this is an interesting possibility there are no reliable figures against which to test it. It is probably true that *T. vaginalis* can produce a symptomless infection in the male and that the treated female can be reinfected in this way. Most venereologists would, I think, agree that such conditions as urethritis and prostatitis occasionally result from infection with *T. vaginalis*.

Possibly improvements in the antigen employed in the test described above would lead to an increase in the titre obtained with positive sera and, if not entirely excluding non-specific agglutination, at least make the distinction between true and false positives easier.

Although it is too early to draw conclusions, the results obtained suggest that the method described merits further study in relation to the problem of human trichomonas infections.

My thanks are due to Dr. A. O. Ross, Dr. D. Lehane, and Dr. E. G. Hall, who provided me with the sera for test.

References

Fuller, A. T. (1938). *Brit. J. exp. Path.*, **19**, 130.
Johnson, G., and Trussell, R. E. (1943). *Proc. Soc. exp. Biol., N.Y.*, **54**, 245.
Kerr, W. R., and Robertson, M. (1941). *Vet. J.*, **97**, 351.
Lancefield, R. C. (1933). *J. exp. Med.*, **57**, 571.
Maxted, W. R. (1948). *Lancet*, **2**, 255.
Middlebrook, G., and Dubos, R. J. (1948). *J. exp. Med.*, **88**, 521.
Muniz, J. (1950). *Hospital, Rio de J.*, **37**, 199.
Pierce, A. E. (1947). *Lab. J.*, **8**, 238.
Riedmüller, L. (1932). *Schweiz. Arch. Tierheilk.*, **74**, 343. Quoted by Trussell, R. E. (1947).
Robertson, M. (1941). *J. Path. Bact.*, **53**, 391.
Tokura, N. (1935). *Igaku Kenkyu, Fukuoka, Japan*, **9**, 1. Abstracted in *Zbl. Bakt., I. Abt. Ref.* (1936), **121**, 149.
Trussell, R. E. (1946). *J. Parasit.*, **32**, 563.
—— (1947). *Trichomonas Vaginalis and Trichomoniasis*. Oxford.
—— Wilson, M. E., Longwell, F. H., and Laughlin, K. A. (1942.) *Amer. J. Obstet. Gynec.*, **44**, 292.
Wendlberger, J. (1936). *Arch. Derm. Syph., Berl.*, **174**, 583.

J. clin. Path. (1952), **5**, 281.

THE RELATIONSHIP BETWEEN IRON STORAGE AND ANAEMIA

BY

W. M. DAVIDSON AND R. F. JENNISON

From the Department of Clinical Pathology, King's College Hospital, London

(RECEIVED FOR PUBLICATION OCTOBER 31, 1951)

In addition to the iron of the haemoglobin and other pigments in active use in the body, there is a quantity stored as ferritin and haemosiderin in the parenchymal and reticulo-endothelial cells. The iron protein complex, ferritin, is difficult to demonstrate as it occurs as a diffuse non-particulate material, but haemosiderin can readily be seen as it forms golden granules which give an iron reaction with the ordinary histochemical methods.

It has been suggested that of these two compounds ferritin is the more labile and readily takes part in the synthesis of haemoglobin, whereas haemosiderin is the reserve store formed, either as the result of denaturation of haemoglobin or by polymerization when there is adequate ferritin already present (Granick, 1949). Thus the readily demonstrable haemosiderin should reflect the amount of ferritin hidden in the cells. Any estimate of these stores of iron would obviously be important in elucidating the cause of many anaemias, and with this in view an attempt has been made to find the relationship between the quantity of readily demonstrable iron in the bone marrow and the various types of anaemia. As the marrow aspirates are sectioned as a routine in this laboratory, the addition of a Prussian blue stain for iron has not entailed much extra labour and such preparations have been used throughout the present work. So far 181 satisfactory specimens from 165 patients have been analysed, 72 with simultaneous serum iron estimations.

Rath and Finch (1948) studied the iron content of the sternal marrow in cover-slip preparations stained by a Berlin blue method, and since the publication of their work we have also tried this method, but in our hands the results have not been nearly as precise as those obtained from sections. When there are large numbers of iron-containing cells they are readily found in squash preparations and even in smears, but the difficulty arises in the negative or near-negative cases. Even with traces of iron, marrow sections give clearly positive results because of the undisturbed cell arrangement.

In contradiction to the findings of Masshoff and Gruner (1951) we have found that the marrow iron and the type of anaemia are related in as far as iron metabolism is involved. On their findings, three quarters of the cases they investigated would fall into our miscellaneous group in which there is no such direct relationship.

Material and Methods

Marrow was aspirated from the iliac crest, sternum, or vertebral spines of the tibia using a Salah needle. The material was put into a watch glass and without loss of time particles of marrow were transferred to slides with a pipette and smear and squash preparations made. The remainder was allowed to coagulate, then freed at the edge to allow adequate contraction of the clot and after about half an hour transferred to Formol-Zenker fixative. The clot was fixed within 12 hours, and after removing the mercuric chloride it was dehydrated, embedded in paraffin, and sectioned.

Smears were stained by Jenner–Giemsa or May–Grunwald–Giemsa techniques and sections by haematoxylin and eosin, Prussian blue, and other methods as indicated. In the Prussian blue method used, equal quantities of 2% potassium ferrocyanide and 2% HCl were mixed and brought almost to boiling point before being applied to the section. The stain was allowed to act for three minutes, and, after washing, the preparation was counterstained with 1% aqueous eosin.

The amount of iron present in the sections was graded as follows: (0) absolutely no intracellular iron demonstrable in adequate sections (at least two particles of cellular marrow sectioned, each not less than 1/3 mm. in diameter, i.e., more than filling the 1/6 in. field of the microscope); (1) only an occasional cell containing iron; (2) 4–6 iron-containing cells per 1/6 in. field; (3) 7–10 iron-containing cells per 1/6 in. field; (4) many cells distended with iron pigment; (5) cells aggregating into iron-containing patches; (6) patches so large that they dominate the field. All marrows do not fall into these clear-cut categories, but to avoid unnecessary subdivision we have generally allotted the marrow to the higher grade. The normal grade is about 2. Grade 6 probably occurs only in heavily transfused patients. Figs. 1–4 illustrate the main grades.

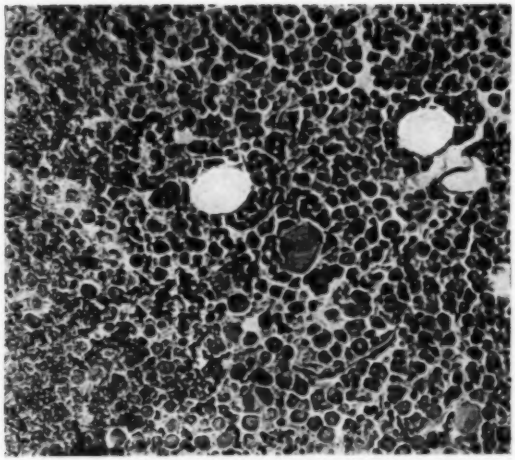


FIG. 1.—Grade 0: hypochromic anaemia; marked hyperplasia.

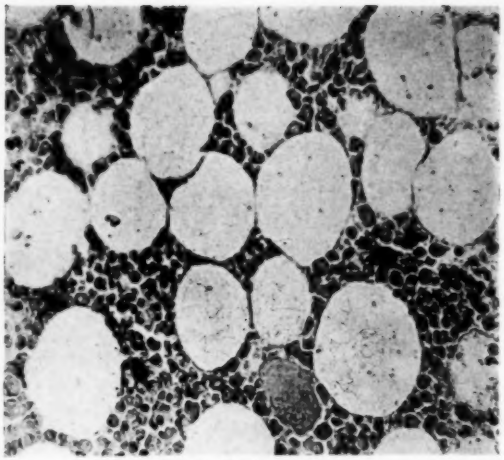


FIG. 2.—Grade 2: normal.

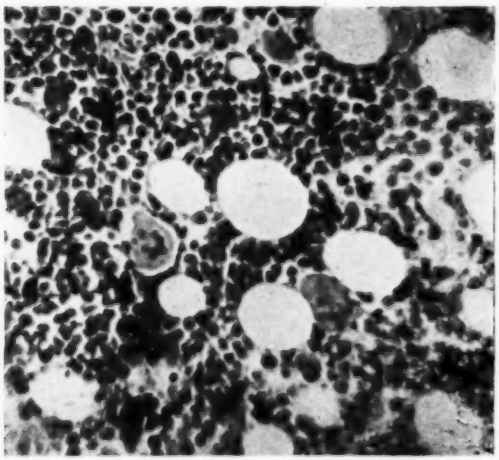


FIG. 3.—Grade 3: chronic infection; slight hyperplasia.

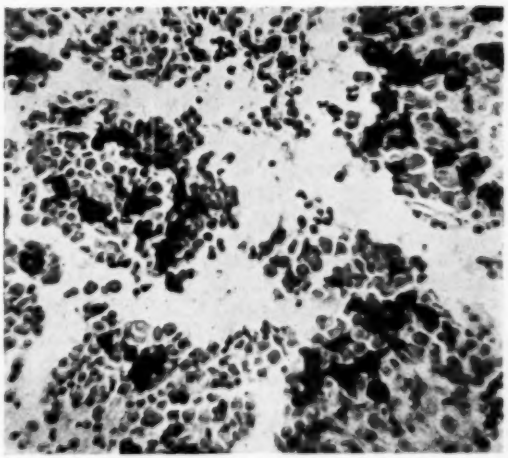


FIG. 4.—Grade 5: haemolytic anaemia; multiple transfusions.

FIGS. 1–4.—Marrow sections (× 260) stained Prussian blue.

The serum iron was estimated by a method similar to that used by Dahl (1948). Employing iron-free glassware throughout, 5 ml. of serum was incubated at 37° C. for one hour with 2.5 ml. of 5% HCl to free the iron from its protein complex. Then 5 ml. of 20% trichloracetic acid was added and allowed to act for two hours. The tubes were centrifuged to deposit the precipitated proteins and 5 ml. of the supernatant transferred to another tube. Concentrated HNO_3, 0.14 ml., was added followed by 1.4 ml. of 5N KCNS and the colour read at once in a photo-electric absorptiometer using an Ilford spectral filter "604." The normal finding by this method is 130 ± 30 μg. per 100 ml.

The normal marrows studied were obtained from patients who proved to have no disorder of the blood or blood-forming organs.

Results

Table I shows the findings in all the marrows examined. It will be seen that the cases tend to fall into four groups. First, the megaloblastic anaemias which are independent of iron metabolism: if iron absorption is normal and there is no blood loss, iron tends to accumulate as a result of diminished erythropoiesis. Usually the serum iron reflects this accumulation and indicates a high percentage saturation of the total iron-combining capacity of the serum.

The second group includes cases of chronic blood loss or failure to absorb iron. These in different ways result in a depletion of the marrow iron stores and a lowering of the serum iron level.

Thirdly are those cases with anaemia due to a toxic cause such as a chronic sepsis or uraemia. Here iron is not used because of depressed erythropoiesis, often associated with a disturbance of protein metabolism, or is not available for haemoglobin formation through an increased affinity of the tissues for iron, as has been suggested by Greenberg, Ashenbrucker, Lauritsen, Worth, Humphreys, and Wintrobe (1947). Despite the presence of stored iron these cases tend to have a low serum iron content.

TABLE I

SURVEY OF ALL MARROWS EXAMINED

	No.	Grade						
		0	1	2	3	4	5	6
Apparently normal	11	—	4	5	2	—	—	—
Haemochromatosis	1	—	—	—	—	1	—	—
Pernicious anaemia and/or subacute combined degeneration:								
Untreated	33	3	7	6	8	9	—	—
Treated	9	2	5	1	1	—	—	—
Other megaloblastic anaemias	8	—	3	—	1	4	—	—
Iron deficiency anaemia:								
Untreated	32	30	2	—	—	—	—	—
Treated orally	4	4	—	—	—	—	—	—
Treated intravenously	3	—	2	1	—	—	—	—
Polycythaemia vera (chronic)	4	3	1	—	—	—	—	—
Steatorrhoea	3	3	—	—	—	—	—	—
Refractory hypochromic anaemia	3	—	—	2	1	—	—	—
Pellagra	1	—	—	—	1	—	—	—
Chronic infection	10	—	1	—	2	5	2	—
Bacterial endocarditis	2	—	1	1	—	—	—	—
Uraemia	5	—	1	2	1	1	—	—
Malignant disease	5	1	1	1	1	1	—	—
Reticuloses	5	2	1	—	1	—	—	1
Cirrhosis of liver	1	—	1	—	—	—	—	—
Multiple myeloma	3	1	—	1	1	—	—	—
Hyperglobulinaemia	1	—	—	1	—	—	—	—
Leukaemia	21	2	7	5	4	1	2	—
Neutropenia	2	—	1	1	—	—	—	—
Haemolytic anaemia:								
Acquired	2	—	—	—	1	—	1	—
Congenital	2	1	—	1	—	—	—	—
Nocturnal haemoglobinuria	1	1	—	—	—	—	—	—
Erythroblastosis	2	1	1	—	—	—	—	—
Thrombocytopenia	1	—	1	—	—	—	—	—
Congenital porphyria	2	1	—	—	—	1	—	—
Porphyrinuria (? alcoholic)	1	—	—	—	—	—	1	—
Still's disease	1	—	—	1	—	—	—	—
Gaucher's disease	1	—	—	1	—	—	—	—
Splenomegaly	1	1	—	—	—	—	—	—
Total	181							

Finally comes the group of miscellaneous conditions which show no characteristic relationship. In most of these any changes in the iron content can be correlated with the presence of other factors bringing them into line with the second and third groups.

Discussion

The Megaloblastic Anaemias.—In general iron is present in this group. The five exceptions were investigated before serum iron estimations were made, but the patients' histories suggest an associated iron deficiency. One was from a woman of

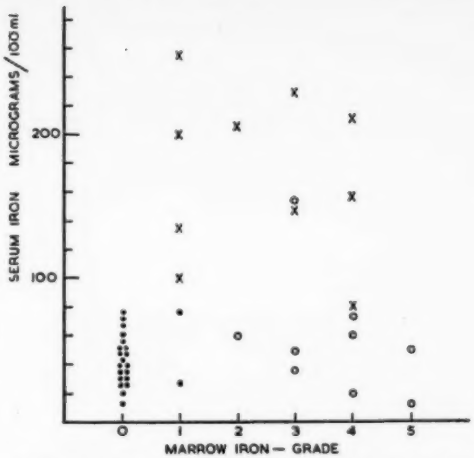


FIG 5.—Diagram showing relationship between serum iron and marrow iron. ● untreated hypochromic anaemias, idiopathic or due to chronic haemorrhage × untreated pernicious anaemia. ○ chronic infection and uraemia of more than three weeks' duration.

40 who in addition to pernicious anaemia had had severe menorrhagia for some years. The pernicious anaemia responded adequately to vitamin B_{12}, but a satisfactory haemoglobin level was only maintained after hysterectomy. Another patient had lived on a "bread and tea" diet for many years. The third and fourth marrows (in the treated and untreated lines) were from the same woman who had subacute combined degeneration with a megaloblastic marrow and a M.C.H.C. of 29%. This patient required iron therapy as well as vitamin B_{12}. The fifth specimen was from a man with subacute combined degeneration. It was taken after treatment; the initial level had been grade 2. Apart from the case which remained at grade 0, and *G.Fi.* (Table II) in whom the amount of iron present on both occasions was very small indeed, all patients from whom satisfactory marrows were obtained before and after treatment showed a drop in iron content. Examples are:

Mrs. H.—In 10 weeks there was a fall from grade 4 to grade 1 while the haemoglobin rose from 6.9 g. to 11.4 g. per 100 ml.

Mrs. D.—In two weeks iron content dropped from grade 3 to 1 with a rise from 7.1 g. to 11.7 g. per 100 ml.

It is interesting that among the untreated patients five of the seven specimens in grade 1 came from women of menopausal age who had been treated with oral iron before pernicious anaemia was diagnosed. Four of these, *E.A.*, *G.Fi.*, *M.F.*, and *W.Y.*, also appear in Table II. The three specimens under "other megaloblastic anaemias" in grade 1 were all from a patient with recurrent pernicious anaemia of pregnancy occurring in the puerperium, and it is possible that the pregnancy depleted the iron store in spite of oral iron therapy.

TABLE II
MEGALOBLASTIC ANAEMIAS

	Haemoglobin (g./100 ml.)	M.C.H.C. (%)	Serum Iron (μg./100 ml.)	Marrow Iron (Grade)
W.H. Pernicious anaemia	6·8	34	80	4
H.B. " "	8·3	31	156	4
B.T. " "	4·0	33	210	4
M.H. " "	5·9	31	144	3
H.E. " "	6·8	32	228	3
E.F. (1) " "	6·2	29	200	2
(2) " "	13·0	30	98	1
E.A. " "	11·2	36	252	1
G.Fi.(1) " "	7·1	31	225	1
(2) " "	12·2	30	77	1
M.F. " "	6·8	35	199	1
W.Y. " "	6·8	31	132	1
W.P. Subacute combined degeneration	13·6	33	97	1
F.R. Nutritional	4·1	32	69	3
L.Ho.(1) Macrocytic anaemia of pregnancy	8·9	34	184	1
(2) " "	11·5	33	97	1
C.K. (1)	3·0	30	330	2
(2)	7·1	27	82	1

The Hypochromic Anaemias.—It is probable that ordinary hypochromic anaemia only develops after the exhaustion of the iron store, although as some of the haemosiderin iron is difficult to mobilize, traces might be found in early cases, but we have not seen this stage. The two patients with simple hypochromic anaemia with a small amount of iron in the marrow (Table III) were relapsing after intravenous therapy. *C.S.* had been given 0.975 g. of "ferrivenin" six months previously which brought his haemoglobin to 13.5 g./100 ml., but he had relapsed. A further complication was that he had received deep *x*-ray therapy for ankylosing spondylitis. *G.P.* had had a transfusion of 2 pints of blood three months previously.

The two cases with the highest serum iron values (82 and 95 μg./100 ml.), *M.Lu.* and *P.G.* (Table III), had already started oral iron when the specimens were taken.

It is interesting that none of the four patients treated with oral iron had developed iron deposits (Table I). One, a man with chronic haemorrhage, had been treated with oral iron for six months during which time his haemoglobin rose from 5.1 g. to 14.8 g. The other three cases also appear in both tables. *J.A.* was treated for 10 months and the haemoglobin rose from 6.0 g. to 17.4 g./100 ml.; *E.K.* for four months with a rise from 9.4 g. to 13.2 g./100 ml.; and *M.G.* for several months without response. The low serum iron in the last patient suggested a failure of absorption and this was confirmed by the response to intravenous iron therapy. The two specimens graded 1 after treatment (Table I) were from a man whose haemoglobin level had risen from 6.0 g. to 10.5 g./100 ml. after blood transfusi n and "ferrivenin" therapy. The marrow in grade 2 was from a patient who had 1.2 g. "ferrivenin" with a rise in haemoglobin level from 8.7 g. to 15.7 g./100 ml. Although the number of treated cases shown here is still small, the results agree with the experimental findings of other workers that oral iron corrects the anaemia in iron deficiency states if absorption is normal or increased as it often is in these cases (Copp and Greenberg, 1946), but once the anaemia is corrected iron absorption ceases and the stores remain depleted (Fontès and Thivolle, 1936). Finch, Hegsted, Kinney, Thomas, Rath, Haskins, Finch, and Fluharty (1950) working with animals have shown that oral iron tends to increase the iron content of the liver first and the reticuloendothelial system later, whereas intravenous iron seems to accumulate to a greater extent in the R.E.S.

TABLE III
HYPOCHROMIC ANAEMIAS*

	Haemoglobin (g./100 ml.)	M.C.H.C. (%)	Serum Iron (μg./100 ml.)	Marrow Iron (Grade)
F.A. Idiopathic†	6·2	24	33	0
K.S. "	7·1	24	36	0
M.Lo. "	4·4	22	37	0
M.J. "	5·6	24	40	0
E.I. "	3·9	25	40	0
M.P. "	13·0	30	48	0
G.S. "	5·9	25	50	0
E.M. "	3·0	21	50	0
M.G. "	5·6	24	60	0
M.R. "	6·2	24	67	0
E.Da. "	7·2	25	68	0
M.Lu. "	6·3	22	82	0
L.P. Chronic haemorrhage	6·9	25	13	0
G.R. " "	7·8	25	19	0
B.T. " "	6·0	24	30	0
N.M. " "	5·4	25	32	0
L.Ha. " "	4·2	25	38	0
D.Q. " "	5·4	26	44	0
E.De. " "	6·0	28	47	0
B.W. " "	5·1	29	57	0
J.B. " "	9·6	28	74	0
E.By. Steatorrhoea ..	9·3	26	45	0
P.G. Ulcerative colitis ..	7·2	25	95	0
W.K. Nocturnal haemoglobinuria	6·3	26	80	0
J.A. Chronic haemorrhage (treated—oral iron)..	17·4	—	200	
E.K. Idiopathic (treated—oral iron)	13·2	26	71	0
M.G. " "	11·1	29	26	0
G.P. Chronic haemorrhage (previous transfusion)	5·4	24	36	1
C.S. Chronic haemorrhage (previous "ferrivenin")	5·7	24	72	1
K.P. Refractory hypochromic anaemia ..	3·0	25	244	2

* Microcytic or normocytic with M.C.H.C. below 30%.
† Term applied to all cases in which there was no demonstrable blood loss, despite the fact that in a few instances no attempt was made to demonstrate the achlorhydria.

Two patients in Table III require explanation. *W.K.*, who had had nocturnal haemoglobinuria for seven years, had such large losses of haemoglobin in the urine during the attacks that he had used up his iron store. *K.P.*, a baby girl, presented at 6 weeks

with a severe anaemia (haemoglobin 3.0 g. %) which showed all the features of iron deficiency. Treatment with oral iron had no effect, as was to be expected, because the serum iron estimations and the iron content of the hyperplastic bone marrow showed that there was no shortage of iron. The child has been kept alive by transfusions and has developed normally, apart from bone changes due to marrow hyperplasia.

Anaemias Due to "Toxic" Causes.—In these (Table IV) the iron stores are normal or increased except in two patients with associated chronic haemorrhage, and one early case of bacterial endocarditis with very little anaemia. In all but one (*G.C.*, suffering from uraemia) the serum iron is well below normal levels, indicating the increased tendency to transfer iron from the serum to the tissues.

TABLE IV

TOXIC ANAEMIAS CAUSED BY CHRONIC INFECTION OR URAEMIA*

	Haemoglobin (g./100 ml.)	M.C.H.C. (%)	Serum Iron (μg./100 ml.)	Marrow Iron (Grade)
P.V. Infection and haemorrhage	11·4	30	47	1
W.G. Infection ? fibroid pulmonary tubercle	9·9	30	49	5
L.T. Infection, liver abscess	8·4	31	61	4
M.D. Infection, gangrene of leg	6·0	30	20	4
G.Fa. Infection, P.U.O.	10·5	32	39	3
N.D. Infection, polyarteritis nodosa	7·5	33	77	4
M.S. Infection with pellagra	7·5	31	49	3
I.P. Infection, bacterial endocarditis	13·8	35	90	1
W.T. Infection and uraemia—pyelonephritis	6·9	32	13	5
G.C. Uraemia	6·9	32	154	3
F.H. ,, ,,	9·6	36	59	2
W.S. ,, and haemorrhage	8·4	29	13	1

* Anaemia in cases with chronic infections of usually more than three weeks' duration and chronic uraemia (blood urea higher than 100 mg. %).

Miscellaneous Group.—In the miscellaneous group the leukaemias show wide variations, in part due to the fact that the iron-containing cells are sometimes difficult to see because of the infiltration. One of these patients *G.R.* (grade 5) had severe haemolytic anaemia of some weeks' duration, while at the other extreme *G.B.* (grade 0) had recently been pregnant. Similarly, in the reticuloses group the iron is obscured, but one patient with haemolytic anaemia treated by multiple transfusions reached grade 6. One patient with congenital porphyria (*M.P.*, Table III) had mild hypochromic anaemia of dietetic origin and no iron in the marrow, whereas the other (*G.L.*, Table V) had haemolytic anaemia and grade 4 iron. *G.R.*, the patient with acquired haemolytic anaemia with grade 5 iron, had had many blood transfusions before the marrow examination was carried out.

TABLE V

MISCELLANEOUS

	Haemoglobin (g./100 ml.)	M.C.H.C. (%)	Serum Iron (μg./100 ml.)	Marrow Iron (Grade)
D.D. Leukaemia—chronic	12·0	31	86	2
J.T. ,, ,,	4·5	32	100	3
M.R. ,, ,,	14·1	33	113	1
L.F. ,, ,,	13·5	32	85	1
E.Bn. ,, ,,	9·0	31	162	1
G.B. ,, acute	7·1	36	112	0
G.R. ,, ,, with haemolysis	3·0	35	328	5
H.D. Acquired haemolytic anaemia	7·1	36	154	3
A.Sm. Carcinoma of pancreas and haemorrhage	6·3	31	59	1
G.L. Congenital porphyria with haemolysis	12·3	35	140	4
A.St. Haemochromatosis	13·1	31	357	4
G.W. Normal—apparently	14·5	32	141	2

Summary

The amount of haemosiderin visible in sections of the bone marrow, the serum iron level, and the type of anaemia have been correlated in a series of consecutive haematological cases.

On this basis the patients have been divided into four groups, three with definite characteristics. In iron deficiency anaemia there is no store of iron in the marrow, even after successful oral iron therapy, and the serum iron is low: in pernicious anaemia the marrow and serum iron levels are raised but fall during treatment: in the anaemia of sepsis and uraemia the marrow iron is high but the serum iron is low; in the miscellaneous fourth group the marrow iron level depends in the main on whether there has been concomitant blood loss, sepsis, or haemolysis, while the highest levels are found in patients treated by repeated blood transfusions. Certain apparent variations have been explained.

The findings suggest that the routine use of the combined marrow and serum iron estimations provides a valuable weapon in investigating cases of anaemia and even in assessing the prognosis in some instances. It is of particular use in determining the part played by chronic sepsis in anaemia, and in localizing a failure in iron metabolism to intake, absorption, excessive loss, or defective utilization.

We wish to thank Mr. G. A. Harwood for the histological preparations.

REFERENCES

Copp, D. H., and Greenberg, D. M. (1946). *J. biol. Chem.*, **164**, 377.
Dahl, S. (1948). *Brit. med. J.*, **1**, 731.
Finch, C. A., Hegsted, M., Kinney, T. D., Thomas, E. D., Rath, C. E., Haskins, D., Finch, S., and Fluharty, R. G. (1950). *Blood*, **5**, 983.
Fontès, G., and Thivolle, L. (1936). *Sang*, **10**, 144.
Granick, S. (1949). *Bull. N.Y. Acad. Med.*, **25**, 403.
Greenberg, G. R., Ashenbrucker, A., Lauritsen, M., Worth, W., Humphreys, S. R., and Wintrobe, M. M. (1947). *J. clin. Invest.*, **26**, 121.
Masshoff, W., and Gruner, P. (1951). *Acta haemat., Basel*, **5**, 19.
Rath, C. E., and Finch, C. A. (1948). *J. Lab. clin. Med.*, **33**, 81.

J. clin. Path. (1952), **5**, 286.

THE EFFECT OF PHOTO-ELECTRIC CELL TEMPERATURE ON FLAME PHOTOMETER DETERMINATIONS

BY

A. O. WILSON

From the Royal Hampshire County Hospital, Winchester

(RECEIVED FOR PUBLICATION JANUARY 8, 1952)

Flame photometers are being more and more widely used for the measurement of sodium and potassium concentrations in blood and other biological fluids. A number of papers have been published describing various types of instrument and the factors which affect their performance (Berry, Chappell, and Barnes, 1946; Domingo and Klyne, 1949; Hald, 1947; Hospadaruk, Frankenberg, and Neufeld, 1951; Natelson, 1950; Proehl and Nelson, 1950; Spencer, 1950; Weichselbaum, 1949), but few of them (Boon, 1945; Fox, 1951; Hald, 1951; Ryssing, 1951; Spencer, 1950) mention the fact that heating the photocells is an important factor influencing the results obtained. It is therefore considered that attention should be directed again to the " heating effect."

The photometer on which this effect was investigated is a modification of that described by Domingo and Klyne (1949). A " megatron " 45 mm. selenium cell replaces the gas-filled caesium cathode cell for the determination of potassium, and the photocells are incorporated in the side-arms of the chimney in order to make the instrument more compact; in spite of the distance of the photocells from the burner (8 in.) they become heated and produce less current. This is particularly important when potassium determinations are being made, since the photocell used for potassium responds less rapidly than the cell used on the sodium side and the duration of one potassium determination is about three times that of a sodium estimation. Proehl and Nelson (1950) found the determination of potassium more difficult than that of sodium on account of fluctuations. When the standard solutions are put through after the unknown, abnormally low readings are obtained for them and the concentration of sodium or potassium is too high. Conversely, if the unknown is put through after the standard solutions the concentration is found to be too low.

By fixing a thermometer to the outside of the photocell carrier it has been possible to demonstrate the relation between variations in the temperature and the galvanometer readings. The following results refer to a standard solution containing 2.5 mg. of sodium per 100 ml., but similar results are obtained with potassium solutions. Without heat insulation between the cell carrier and the rest of the side-arm the galvanometer readings remain steady for only the first few minutes after lighting,

TABLE

DIFFERENT RESULTS OBTAINED WHEN AN UNKNOWN SOLUTION (DILUTED SERUM) IS PUT THROUGH BEFORE AND AFTER THE APPROPRIATE STANDARD SOLUTIONS*

Solution Sprayed	Galvo. Reading	Concentration Calculated for Unknown Serum
X Serum, diluted 1/10	11·3	
↓ Standard, 2·0 mg K%	13·5	17·4 mg K%
↓ Standard, 1·5 mg K%	9·2	16·7 mg K%
Y Serum	10·7	
X Serum	10·3	
↓ Standard, 2·0 mg K%	12·2	17·7 mg K%
↓ Standard, 1·5 mg K%	8·0	16·8 mg K%
Y Serum	9·5	

*One series of readings taken between 10 and 20 minutes after lighting, without heat insulation. Approximately 2 minutes elapsed between the reading marked X and Y in each group. The serum had a potassium content of 18·0 mg.% determined chemically.

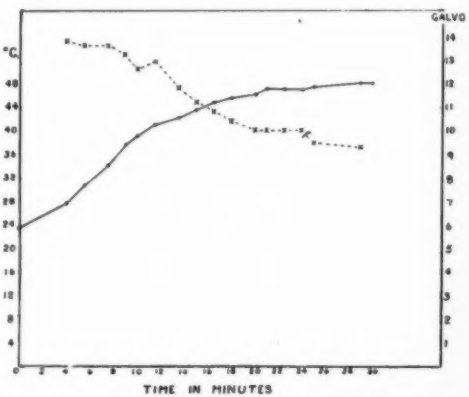


FIG. 1.—Photocell incorporated in the side-arm without heat insulation. For key, see next page.

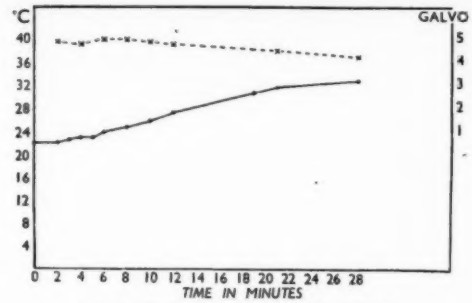


FIG. 2.—Photocell carrier partially insulated.

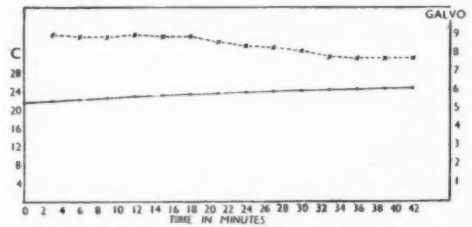


FIG. 3.—Cell carrier completely insulated from side-arm.

•——•——• Temperature.
×- - -×- - -× Galvanometer reading.
(Sodium chloride solution 2·5 mg. Na%.)

and then fall rapidly during the succeeding 15 minutes. Thereafter the readings continue to fall slowly. The main temperature rise occurs during the 15-minute period of rapid fall in reading (Fig. 1). Partial heat insulation makes the readings much more constant (Fig. 2), and was effected by the introduction of a thick cardboard collar between the side-arm and the cell carrier. Complete isolation of the cell carrier from the side-arm and the introduction of a $\frac{1}{2}$-inch air gap, surrounded by a thick black cloth, gives readings (Fig. 3) which are constant for almost 20 minutes. There follows a period in which there is some falling-off in the galvanometer readings, but these soon settle again.

It is appreciated that this effect has been investigated on only one type of instrument and that it may not apply to instruments of other types.

Summary

Attention is drawn to the effect produced by heating the photocells in a flame photometer, and to the influence on results.

I wish to thank Dr. C. Wrigley for the provision of the laboratory facilities for this investigation, and I must acknowledge my indebtedness to Mr. P. W. Ingram for defraying part of the cost of the work. I also wish to thank Mr. C. Lambert for his valuable assistance.

REFERENCES

Berry, J. W., Chappell, D. G., and Barnes, R. B. (1946). *Industr. Engng Chem. (Anal. Ed.)*, **18**, 19.
Boon, S. D. (1945). *Vlam-fotometrie*. Thesis, Amsterdam.
Domingo, W. R., and Klyne, W. (1949). *Biochem. J.*, **45**, 400.
Fox, C. L. (1951). *Analyt. Chem.*, **23**, 137.
Hald, P. M. (1947). *J. biol. Chem.*, **167**, 499.
Hald, P. (1951). *Meth. med. Res.*, **4**, 79.
Hospadaruk, V., Frankenberg, B., and Neufeld, A. H. (1951). *Canad. med. Ass. J.*, **65**, 264.
Natelson, S. (1950). *Amer. J. clin. Path.*, **20**, 463.
Proehl, E. C., and Nelson, W. P. (1950). Ibid., **20**, 806.
Ryssing, E. (1951). *Scand. J. clin. Lab. Invest.*, **3**, 17
Spencer, A. G. (1950). *Lancet*, **2**, 623.
Weichselbaum, T. E., and Varney, P. L. (1949). *Proc. Soc. exp. Biol., N.Y.*, **71**, 570.

J. clin. Path. (1952), **5**, 288.

TECHNICAL METHODS

A RAPID METHOD FOR DETERMINING FAECAL FAT

BY

O. E. NEUFELD

Repatriation General Hospital, Heidelberg, Victoria, Australia

(RECEIVED FOR PUBLICATION JANUARY 9, 1952)

The percentage of fat (split and unsplit) in faeces gives a useful indication of the efficiency of digestion and adsorption of fat by the intestinal tract. It is a definite help in the diagnosis of pancreatic disease, sprue, and other conditions causing steatorrhoea.

The methods recommended by standard textbooks for the determination of faecal fat are time-consuming, laborious, and extremely unpleasant because they foul the air in the laboratory, and usually three days are required to complete the test. The most odious procedure in the standard method is the drying of the faeces in an open dish to a consistency which permits the sample to be powdered.

The new method requires barely two to three hours from beginning to end. The faeces are dehydrated in a closed system and not even a fume cupboard is required to keep the laboratory atmosphere clean.

In common with the standard methods in this method, low molecular fatty acids, derived mainly from the decomposition of cellulose, are volatilized, ensuring more accurate results for clinical purposes.

The method is equally suitable for the determination of fat in food for the quantitative study of fat balances, when the proportion of fat intake to fat excretion is to be determined. Duplicates of the patient's food are passed through a mincer or another suitable disintegrator and after very thorough mixing the same method is applied.

Method

The wet faeces are treated with an exact amount of concentrated hydrochloric acid to liberate free fatty acids from soaps. They are then subjected to a distillation process using xylene to carry the water to a calibrated part of the apparatus, where it can be accurately measured. The fats and fatty acids go quantitatively into the xylene solution. After purification by means of petrol ether, the fatty residue is weighed and the amount of free fatty acid determined in the usual way by titration with alcoholic standard alkali.

Reagents.—The following are required: concentrated hydrochloric acid A.R.; xylene (any pure or commercial grade, boiling between 130–150° C., is suitable); petrol ether (boiling range 40–60° C.); N/10 sodium or potassium hydroxide solution.

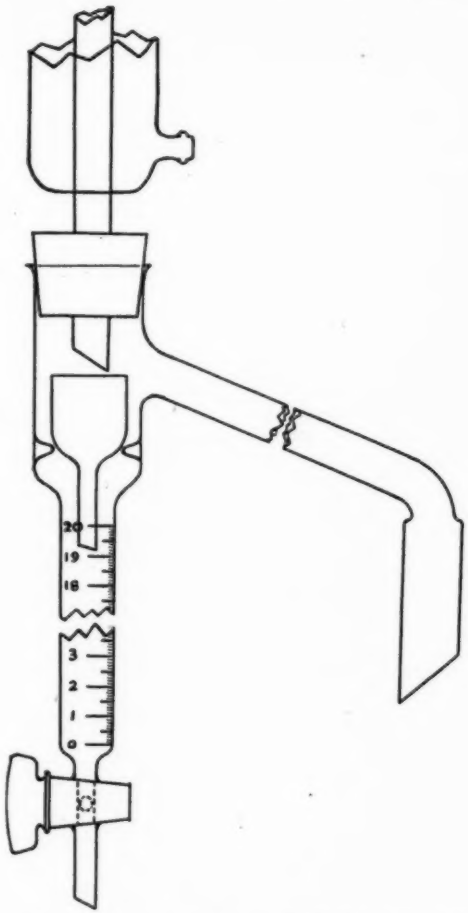
Apparatus.—The apparatus used for the determination of water is shown in the illustration. It can easily be made from part of a burette. The reflux condenser may be connected to the apparatus by a standard ground joint, but a good cork is much preferable for connecting the flask since ground joints at this place show a tendency to stick badly.

The little funnel, which prevents droplets of water floating back with the returning xylene, rests loosely on three or four indentations put into the wider part of the apparatus.

The distance of the flask from the measuring part of the apparatus should not be less than 6 to 8 in. to avoid excessive heat radiating on this part. The tube connecting the apparatus to the flask is wide enough to give ample room to the glass rod, protruding from the flask during the distillation.

Procedure.—The specimen is thoroughly mixed until it is homogeneous, when 15–18 g. is transferred through a wide-neck funnel into a 500 ml. wide-neck flask avoiding smearing any of the specimen on to the sides of the flask. A glass rod protruding about 1–1½ in. above the neck of the flask is tared with the flask. Exactly 3 ml. of concentrated hydrochloric acid is added and well mixed with the faeces by means of the glass rod, which is left in the flask during the distillation. About 200 ml. of xylene is added and the flask is connected to the apparatus. The distillation is carried out at a moderate speed (three to six drops per second) until no further water separates in the graduated part of the apparatus, usually requiring 25 to 45 minutes. With care, faeces can be heated with the xylene directly on a wire gauze. With food, however, local overheating easily causes scorching and a paraffin oil bath, heated to about 150–170° C., should be used.

The amount of water distilled is noted, and 2.7 ml. subtracted from this amount, allowing for the 3 ml. of concentrated hydrochloric acid added. The amount of dry faeces can now be calculated.



The total amount of xylene in the flask is determined by weighing the flask (including the glass rod) to the nearest 0.5 g. The weight of the flask and rod plus the amount of dry faeces calculated is subtracted from the total weight. When the approximate fat content of the faeces has been determined, a correction may be made by subtracting the amount of fat from the weight of faeces, since the fatty part is now in the liquid solvent phase.

An aliquot part of the xylene solution (10–20 g. weighed accurately to 0.01 g.) is evaporated to dryness on a water bath by vacuum distillation, air blowing, or air suction. The residue is treated with several small portions of petrol ether making a total of about 10 ml. The petrol-ether extracts are transferred by a Pasteur pipette to a centrifuge tube and the precipitate is spun down. If a stoppered, calibrated centrifuge tube is used, an aliquot part of the petrol-ether solution is used. Otherwise the precipitate is washed with two more portions of petrol ether (2–3 ml.), centrifuging each time. Filtration through filter paper is not advisable due to the difficulty of washing down fat which, by capillary action, collects at the highest part of the filter.

The petrol-ether solution is evaporated on a water bath in a tared flask of about 25 ml. capacity. Last traces of solvent and volatile fatty acids are removed by blowing a current of air (or any other gas available in compressed form at the laboratory) into the flask while it is still hot, spreading the fatty residue to a thin layer by rotating the flask, or keeping the flask under vacuum on a boiling water bath.

When constant weight is obtained, the free fatty acid may be determined by dissolving the residue in warm benzene and titrating the acids in the usual way with alcoholic caustic soda or potash to the end-point with phenolphthalein.

To comply with the standard textbooks 1 ml. of N/10 standard caustic solution is calculated to correspond to 0.0268 g. of fatty acid. The calculation is as follows:

$$\% \text{ fat in dry faeces} = \frac{100\ p\ X_t}{F\ X_a}$$

when p = weight of fat residue from aliquot part of xylene; X_t = weight of total xylene after removal of water; X_a = weight of xylene aliquot taken for fat determination; F = weight of dry faeces calculated from weight of wet faeces minus the amount of water distilled after allowing for the hydrochloric acid added (2.7 ml.).

Summary

A method is described for the determination of fat (split and unsplit) in faeces and food. It requires only two or three hours for a complete analysis. The results are quite accurate enough for all clinical purposes. The method was compared with that of Holt, Courtney, and Fales (1919), and the results agreed very well. The results of the new method were slightly lower (0.2–1% on the dry faeces), less inert material being extracted by the petrol ether than the ether-petrol-ether mixture used in the other method after the addition of a considerable amount of alcohol.

I wish to thank the Chairman of the Repatriation Commission for permission to publish this paper, Dr. R. B. Maynard, head of the Pathology Department, for his interest, and Mr. D. Lijnzaat for technical assistance.

Reference

Holt, L. E., Courtney, A. M., and Fales, H. L. (1919). *Amer. J. Dis. Child.*, **17**, 38, 241, 423; and **18**, 107. Cited in Harrison, G. A. (1947): *Chemical Methods in Clinical Medicine*, 3rd ed., pp. 487–496. London.

J. clin. Path. (1952), **5**, 290.

A SIMPLE METHOD FOR THE ASEPTIC FILLING OF SCREW-CAPPED CONTAINERS

BY

J. E. O'HAGAN

From the Laboratories of the Red Cross Blood Transfusion Service, Brisbane, Australia

(RECEIVED FOR PUBLICATION FEBRUARY 5, 1952)

The increasing use of screw-capped tubes and bottles in bacteriology, following their introduction by McCartney (McCartney, 1933; Mackie and McCartney, 1948), suggests many applications for a method which has been used successfully for dispensing pooled human serum for transfusion. The principle of the method adopted is that the glass container is autoclaved with its cap fitting loosely, but suitably wrapped, the wrappings are removed and the cap pushed into a "gripper" under a hood, while the container is filled under the same hood by transferring its neck to a spring-loaded valve which delivers sterile fluid. The "gripper," a hard rubber ring with a tapered inner hole, is used both to remove and to replace the cap. On filling to the desired level, the valve is shut by lowering the container, which is then screwed up into the firmly held cap. Should it be desired to keep the outside of the cap sterile, this can be achieved by covering the cap with sterile paper before giving a final tightening by hand.

A description of the technique for filling half-litre serum bottles will illustrate the method.

The Apparatus

The hood is constructed as follows (Fig. 1). A 108-oz. clear glass Winchester is cut 10 in. from the top by means of a hot wire glass cutter and the edge ground on a sheet of flat iron fed with a paste of 80 mesh carborundum in 50% aqueous glycerine. A 5¼ in. circle of ¼ in. thick firm rubber (B) is then ground on an emery wheel to fit snugly into the neck of A. On the underside of B a sheet of 16 G. stainless steel or brass (C) is mounted to give rigidity, and then the cap gripper (M) of firm rubber (3 in. diameter, ⅞ in. thick) is attached with four 1-in. wood screws (R). One side of the gripper is ground to allow the valve to operate smoothly with this size bottle and a recess made also to allow the 5-in. carriage bolt (D, with its round head ground flat) to seat properly.

The valve is built in the following way. To a 1½ in. diameter stainless steel or brass disc (Q) with a ½ in. diameter central hole are soldered two stainless steel or phosphor bronze rods on one side, and on the other a rubber washer is secured by means of two small aluminium rivets. This washer may be made in the same mould as the rubber closure which fits inside the cap, but should be harder. The rods pass through bearings made from two worn 11 G. transfusion needles (J) cut off to within ½ in. of the hub and joined by a brass bar at the top. Then, after a 10 mm. glass tube (H) and a suitable spring (I) have been fitted, the rods are soft-soldered to a brass bar at K. A length of 3/16 in. I.D. gum rubber tubing (G) is fixed to H and coiled to fit through the valve assembly, two screw-eyes (not shown) with ½ in. diameter holes being fixed immediately in front of and behind the valve to guide the rubber through. The assembly is then placed in the hood, the rubber tubing (G) attached to the 8 mm. glass tube (F) which, with the carriage bolt (D), is placed through holes in the rubber stopper (E). The brass collar (O) is then fixed and the bolt secured with a washer and nut. A removable spacer bar (L) is inserted through ½ in. holes in B and C to hold the valve mechanism open, and so prevent the rubber tubing from sticking during autoclaving.

The hood with spacer bar in position is rinsed, placed on one layer of lint and two of brown paper, and these are then tied firmly at the neck. The upper end of F is connected by rubber tubing to the large vessel intended to hold the sterile fluid. The vessel and its attachments are then autoclaved, and, after filling the vessel by Seitz filtration, they are conveyed to a "sterile" room, in which is arranged a shelf to hold the vessel, a fixed stand with a suitable clamp to hold the hood by the neck, and a pivoted adjustable rest on which the base of the bottle sits during the filling.

The receiving bottles are prepared, after suitable washing treatment, by rinsing with pyrogen-free distilled water, and, after draining, 3 ml. of similar water is run in. The caps may require slight crimping below the knurling if a large soft rubber closure is to be retained. Each cap, with the rubber closure in position, is then placed loosely on the top of a bottle, turned anticlockwise until it drops as far down as it will go, and then turned a further quarter turn. The caps are covered

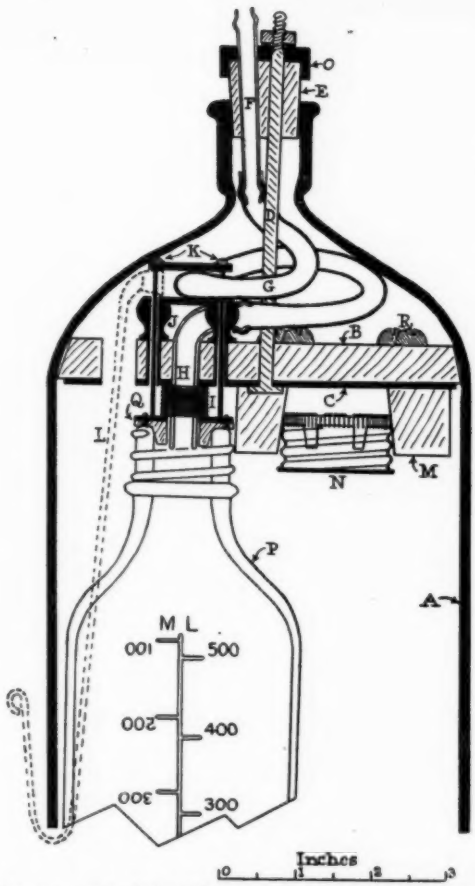


FIG. 1.—A. Top of glass Winchester. B. ¼ in. thick rubber sheet. C. Stainless steel sheet 16 G. D. 5 in. carriage bolt. E. Rubber stopper. F. Glass inlet tube 8 mm. O.D. G. 3/16 in. I.D. amber rubber tube. H. Glass delivery tube 10 mm. O.D. I. Spring. J. Old 11 G. needles. K. Brass bar, soft soldered. L. Spacer bar. M. Rubber "gripper." N. Aluminium cap with rubber closure in place and holes in top for insertion of needles. O. Metal collar. P. Pyrex bottle in filling position. Q. Stainless steel or brass disc. R. Wood screws holding "gripper."

with a layer of lint and two layers of brown paper, which are held in place by means of a strong rubber band placed well down on the neck. The bottles are then autoclaved at 20 lb. per square inch for 20 minutes. Complete drying of the bottle interior is not necessary, since the neck and cap are the last parts to cool (due to the wrappings) and moisture does not condense there.

Filling Procedure

The bottles are then transferred to the "sterile" room. The hood is clamped in position and uncovered. The wrappings of a bottle are removed, without disturbing the cap, by raising an edge with the thumb and forefinger of the right hand while the middle finger rests on the top and the other fingers on the rear of the neck of the bottle (Fig. 2). The cap is pushed up into the "gripper" (Fig. 3) and the bottle transferred to the valve; in the case of the first bottle, the spacer bar is removed and the bottle rest adjusted to the correct height (Fig. 4). Air pressure is then applied to the storage vessel to deliver its contents.

After filling to the desired level the bottle is lowered slightly to cut off the flow and inserted once again in the cap (Fig. 5), into which it is screwed up using both hands on the base of the bottle. A feature of the device is that when the spring (I) expands, the tip of the delivery tube (H) is covered and it is practically impossible for the operator to wet the rim of the bottle and so leave a trail for the entry of micro-organisms. By using a soft rubber closure of the type shown inserted in N, a tight dry seal is maintained at all times. The sloping projections should not be too long, however, or the closure will be drawn down on to the bottle during autoclaving.

If the top of the cap is to be kept sterile it is covered with a sterile double-folded sheet of brown paper, a rubber band placed to hold it, and the cap given an extra tightening by hand. Later the paper may be removed and the cap dipped into a plastic capping solution* to which phenol has been added to a concentration of 4%.

Small volumes of the sterile fluid may be run off at any stage for routine sterility, toxicity, biochemical, or other tests by pushing the lip of a sterile test-tube on the valve. If screw-topped test-tubes† are used and a suitable gripper provided under the hood, "flaming" may be dispensed with altogether, but if cotton-plugged tubes are used it is well to see that the rim is well covered and flamed before and after insertion under the hood.

To clean the hood the spacer bar is replaced and the rubber inlet tube placed on a water jet, followed by rinsing with pyrogen-free water. After using three times, the inner rubber tube (G) should be removed and thoroughly cleaned, being replaced after using six times. Grippers may need occasional replacement. Worn grippers may have a small segment cut from them and are then useful for removing tight caps from used bottles.

Results

At the Blood Transfusion Service laboratories of the Queensland Division of the Australian Red Cross Society over 500 batches, comprising more than 5,000 litres of pooled human serum, have been bottled without one reported contamination. Seitz-filtered media have been filled also by this means and given negative sterility tests. One operator, unaided, can fill 60 half-litre bottles per hour.

* N/F capping solution supplied by the London Capsule Company.
† Culture tubes No. 45066. 20 × 150 mm. made by the Kimble Glass Company.

FIG. 2.—Removing coverings.

FIG. 3.—Removing cap with "gripper."

FIG. 4.—Filling.

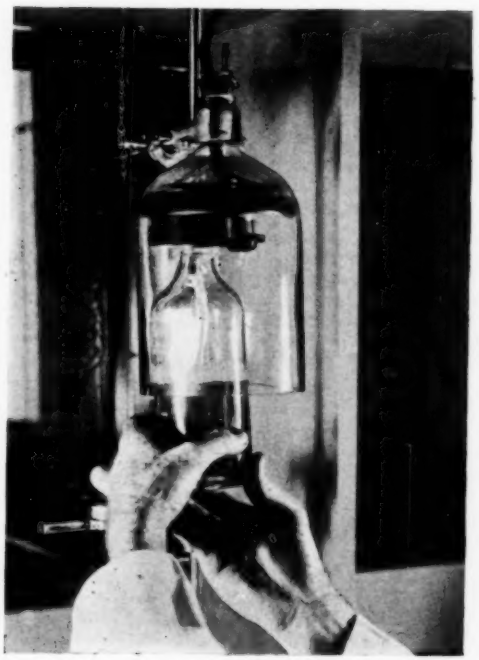


FIG. 5.—Screwing bottle into cap.

Summary

The construction of a device and the technique of a method for the aseptic filling of screw-capped containers is described.

The method is simple, rapid, and convenient, and the conventional flaming procedures may be dispensed with entirely.

No contaminations were reported following the filling of more than 5,000 litres of pooled human serum for transfusion.

I am much indebted to Dr. A. E. Shaw for his continuous encouragement and helpful advice; to Mr. A. Tuffley for the photographs; to the Queensland Rubber Co. for the rubber mouldings; and to Misses H. Crawford and M. Tunn and Mr. M. Robins for the sterility checks.

REFERENCES

McCartney, J. E. (1933). *Lancet*, **2**, 433.

Mackie, T. J., and McCartney, J. E. (1948). *Handbook of Practical Bacteriology*, 8th ed., p. 141. Edinburgh.

J. clin. Path. (1952), 5, 294.

A DIRECT SCANNING APPARATUS FOR READING ELECTROPHORETIC PAPER STRIPS

BY

L. L. GRIFFITHS

From the Department of Pathology, Farnborough Hospital, Kent

(RECEIVED FOR PUBLICATION APRIL 16, 1952)

Since the publication of papers on the electrophoresis of serum proteins in filter paper strips by Cremer and Tiselius (1950), Durrum (1950), Turba and Enenkel (1950), and Flynn and de Mayo (1951), several attempts have been made to simplify the technique by the elimination of the tedious elution and subsequent estimation of the protein fractions. Grassmann, Hannig, and Knedel (1951) rendered the strip translucent with anisol and it was then read in the special apparatus described by Knedel (1951). This is unobtainable in Britain and the method of construction results in a graph of small dimensions which is difficult to analyse. A much simpler apparatus was devised, which, while accurate, had the disadvantage that it was not permanent, but utilized apparatus already in the laboratory without interfering with its use for the original purpose for which it was designed. In a bigger laboratory where more estimations are carried out the need was felt for a more elaborate apparatus, and the one to be described was constructed with this end in view.

The apparatus consists of a wooden box 23 in. long and 19 in. wide and approximately 4 in. high. The top is made of plywood and is covered with a sheet of perspex. In these is cut a slot $\frac{1}{2}$ in. wide and 12 in. long. One inch from the end of the slot is an oblong opening of such a shape as to take the mounting of an EEL 37 × 50 mm. barrier layer photoelectric cell. A second piece of perspex, half the size of the top, is now cut. This has only the long slot cut in it to correspond with the slot in the top of the box. A sheet of cardboard is cut to the same shape and in addition a slit 2 in. long and 1/10 in. wide is also cut out so as to be over the position of the photoelectric cell. Two paper rulers are pasted on the edges of the cardboard, and the exposed cardboard painted black. The smaller piece of perspex is placed over the card and the two bolted to the top of the box so that the three long slots coincide. Two narrow pieces of perspex are cemented to the top 3 in. apart so as to act as guide rails to the glass strip carrier. The lamp housing consists of a perspex box containing a 25 watt 250 volt lamp with a straight slot 2 in. long and $\frac{1}{2}$ in. wide cut in the centre with its long axis at right angles to the guide rails. This opening is filled with a piece of $\frac{1}{2}$-in. perspex rod cut lengthways so as to form a cylindrical lens. The opacity left by the saw can be easily removed by painting the cut surface with acetone or perspex cement. Between the lamp and the lens is mounted a 2 × 2 in. glass filter (Chance yellow OY).

The lamp house is mounted over the 1/10 in. slit so that the cylindrical lens projects a beam of light through it and on to the photoelectric cell. The house should be raised sufficiently so that the bottom of the lens just touches the glass carrier strips.

A single pole, single throw switch, a 20,000 ohm potentiometer and an EEL galvanometer with a logarithmic transmission scale, are mounted in convenient places on the top of the box. Under the box and parallel with the centre of the $\frac{1}{2}$ in. slot runs a $\frac{1}{4}$ in. Whitworth screw 25 in. long, which has a pitch of 0.0200 in. This is held in position by suitable drilled strips of metal. The threaded rod projects for 2 in. through one of the sides and to this is fixed a small handle. A suitable brass nut is soldered to a metal strip which in turn is bolted to a perspex square, the top of which is cut to such a shape that $\frac{1}{2}$ in. projects through the long slot in the top of the box. One of the outer contacts of the potentiometer is wired to the negative terminals of the photoelectric cell and the galvanometer. The other outer contact goes to the positive terminal of the meter. The centre contact of the potentiometer is connected to the positive terminal of the cell. The switch is wired across the two terminals of the metre and when shorted protects the movement. By making the connexions in this manner, a constant resistance is placed across the coil of the meter which therefore does not vary its sensitivity with each setting of the resistance.

The stained electrophoretic strip is rendered translucent and is placed between two glass strips which are 3 × 12 in. These are kept in apposition by "cellotape" and the glass is placed between the guide rails. A portion of the paper strip which has no protein bands is placed in front of the slit and the galvanometer adjusted to 100% transmission or zero by aid of the 20k potentiometer. Two revolutions of the handle move the paper strip 1/10 in. The deflections of the meter are recorded for each movement of 1/10 in. The graph is then plotted and measured by the usual technique. Similar scanners have been constructed with different screw-threads and consequently different slits. In all, slits of 1/32, 1/16, 1/20, 1/10, and 1/12 in. have been used. No advantage has been found from using a very narrow slit. 1/16 in. was found to give an excellent graph which was easy to analyse and to measure but suffered from the slight disadvantage that the size of the graph does not correspond with the separation of the bands when plotted on the usual 1/10 paper. With a 1/10 in. slit the length to be scanned as measured on the built-in rulers corresponds exactly with the graph, and this size of slit has been adopted as standard in this laboratory.

Figs. 1*a* and 1*b* show the construction. None of the dimensions is critical except that the size of the slit must be an exact multiple of the pitch of the screw.

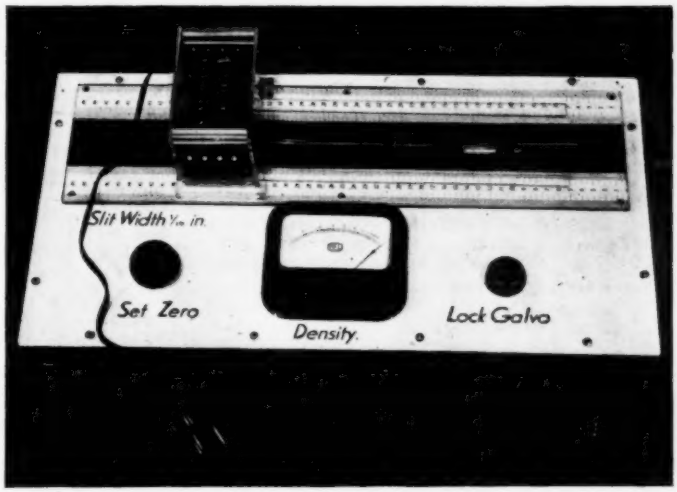


FIG. 1*a*

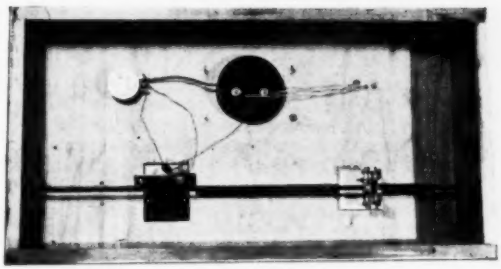


FIG. 1*b*

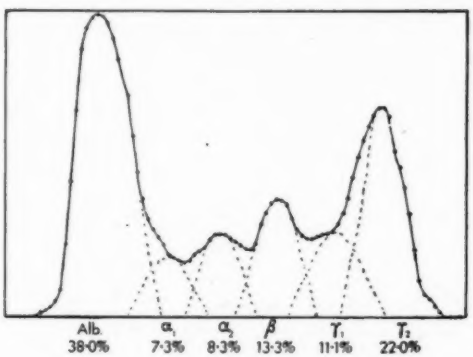


FIG. 2

Fig. 2 is an example of the scanning of a typical strip from a case with increased gamma globulins.

With this apparatus one person can record and plot 100 to 150 points in 10 minutes, and it can be operated by a relatively unskilled worker with ease and accuracy.

My thanks are due to Mr. E. G. Poynter for the three photographs.

REFERENCES

Cremer, H. D., and Tiselius, A. (1950). *Biochem. Z.*, **320**, 273.
Durrum, E. L. (1950). *J. Amer. chem. Soc.*, **72**, 2943.
Flynn, F. V., and Mayo, P. de (1951). *Lancet*, **2**, 235.
Grassmann, W., Hannig, K., and Knedel, M. (1951). *Dtsch. med. Wschr.*, **76**, 333.
Knedel, M. (1951). *Med. Mschr.*, **5**, 707.
Turba, F., and Enenkel, H. J. (1950). *Naturwissenschaften*, **37**, 93.

J. clin. Path. (1952), **5**, 296.

REVIEWS

Pathology of the Cell. By G. R. Cameron. (Pp. xvi+840; 64 illustrations and 41 text figures. £4 4s.) London: Oliver and Boyd. 1952.

Progress in such biological sciences as pathology depends upon their constant fertilization with ideas and methods from physics and chemistry. In the early part of the last century developments in optics made possible the "cellular pathology" of Virchow and the recognition of the role of microbial parasites in the causation of disease. In our day great strides in physical chemistry and biophysics—especially those related to radioactive isotopes and electron microscopy—are opening out vistas in pathology that are quite as extensive as those of 100 years ago. The purpose of the present book is to encourage pathologists to seek acquaintance with these new ideas, and to apply them to their task of unravelling the complexities of disease processes. As a guide to such ideas and as a source book for research workers, the present volume has no equal in pathology; its bibliography alone contains nearly 10,000 references.

No cursory review can do justice to a work such as this which is at once wide in scope and full in treatment. It starts with a broad survey of cytology, traces it from its beginnings with the gradual emergence of the "cell theory" from its early mainly botanical origins, and thence passes to the modern analysis of the structure and function of the individual cell components, the nucleus at rest and in division, the cytoplasm with mitochondria, Golgi apparatus and other organelles, and the external membrane with the agents that affect its permeability. All this mass of information is considered with great thoroughness and with a wealth of detail that will render it of great value to normal cytologists as well as to pathologists.

An interesting interpolation in this portion of the book is a short essay on teleology. Since first minted, this counter has passed through so many hands that most of its original impress has been lost. Yet in spite of its chequered history, the idea still has a place in post-Darwinian biology, as can be seen from the attention that it has received from Adami, T. H. Morgan, Welch, Sherrington, and Boycott. It is a pity that this chapter ends before the possibilities latent in a teleological approach to modern pathological problems has been fully explored.

Although their contents are for the most part furthest divorced from the main cytological theme of the book, the chapters that deal with the "tissue cell"—the various forms of degeneration, necrobiosis, and different potentialities for regeneration—will probably afford the greatest interest to clinical pathologists. The manifold problems of repair in various organs and tissues are considered very fully, and since many problems in current medicine and surgery are intimately concerned with efforts to stimulate various organs and cells to undergo regeneration after injury, the contents of this large section of the book (chapters 23 to 27) are of much practical as well as scientific interest. Many of the principles that have emerged, mainly from laboratory research, are now ripe for trial in human medicine; of all members of the profession, clinical pathologists are probably in the best position to bring such knowledge to practical fruition. The sections on normal and pathological calcification, on liver necrosis and repair, and on the survival of specialized structures after grafting, to all of which the author has made important contributions, are especially notable.

The book ends with a discussion on the possible applications of new biophysical and physico-chemical ideas and techniques to pathology. It seems likely that methods of investigation in which physical procedures are employed will, during the next half-century, revolutionize many of our present biological concepts. In contrast with most biochemical techniques, physical modes of examination can often be employed on living systems with little or no interference with the normal activities of the cells examined. Moreover, they may afford information not only on the nature of the metabolic activities taking place in a tissue but also on their localization in the actual cell organelles concerned. By drawing attention to the possibilities for future biological research that lie in *x*-ray crystallography, polarization optics, surface chemistry, and modern developments in microscopy, the author will encourage a more lively interest amongst pathologists in such procedures as instruments in research.

There can be no doubt that Professor Cameron has added a valuable book to British medical literature. It shows persuasively how closely modern medicine, even in its practical aspect of therapeutics, is integrated with and ultimately dependent upon fundamental biological concepts and methods, and how important it is to maintain the current of research both in physiology and pathology irrespective of its immediately apparent value. At a time when the achievements of applied sciences are so prominently in evidence, it is more than ever important to realize that their roots go down and draw nourishment from a vast matrix of seemingly academic discovery. It is desirable, too, when efforts are being made to force many young medical men into conventional forms of postgraduate training, that some at least of the present generation should remain sympathetic to attempts to build the fabric of knowledge at all its levels. The present volume demonstrates very convincingly how profoundly modern medicine is indebted to a tolerant attitude in the past.

G. PAYLING WRIGHT.

The "Neurility" of the Kidney. A Monograph on Nerve Supply to the Kidney. By C. G. De Muylder. (Pp. 80; 46 text figures. 25s. net.) Oxford: Blackwell Scientific Publications. 1952.

The contributions of Dr. De Muylder to work on the innervation of the kidney are well known, and this monograph is an account of the present state of our knowledge.

The studies comprise and detail an approach to the intricacy of plexuses in the adult kidney from the early embryonic stages in the human and other species. Nerve-staining is more constantly successful in embryonic material, and an added result has been the discovery of structures, hitherto unsuspected, which are present in developmental stages but absent at birth. Thus intravenous nerve-endings present in the embryonic kidney are thought to be possible presso-sensitive areas whose functions are acquired later, possibly by the juxtaglomerular apparatus, as a more complex yet local short reflex regulatory mechanism influencing the afferent arteriolar walls (micromuscular sphincter).

As a general survey the subject is dealt with in five chapters: normal innervation of the kidney; the normal juxtaglomerular apparatus, comparative anatomy, the possible relationship between nervous elements and some aspects of renal pathology, and finally the significance of the nervous constituent of the kidney. The work of the Belgian school is naturally and very clearly outlined and illustrated with fine drawings and photomicrographs from the author's earlier published work. The bibliography is adequate and suitable for those interested in the subject. The English of the text is excellent and engagingly un-English in places to remind us of the origin of the volume. The format and finish are of Blackwell's usual high standard.

J. F. HEGGIE.

J. clin. Path. (1952), **5**, 298.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the abstracting journal,* ABSTRACTS OF WORLD MEDICINE, *published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Clinical Experiences with Aureomycin in Surgical Infections. RUTENBERG, A. M., SCHWEINBURG, F. B., and FINE, J. (1951). *Ann. Surg.*, **133**, 344.

Aureomycin was administered by mouth (1.5 to 3 g. daily), intramuscularly (later abandoned), and intravenously (600 to 1,500 mg. daily) to 263 surgical patients. The authors reached the following conclusions: (1) Aureomycin was superior to streptomycin and phthalylsulphathiazole for pre-operative preparation of the large intestine. (2) It was a satisfactory agent for the prevention of post-operative complications. (3) It was efficient when combined with surgery in the treatment of peritonitis. (4) The acute exacerbations of ulcerative colitis were not influenced by the drug. (5) Of 89 patients with urinary infections 73 responded to aureomycin. In the others the urine was not sterilized, although there was clinical improvement. The resistant organisms were *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Bacterium coli*, and *Aerobacter aerogenes*. (6) Aureomycin was of value in the treatment of infections associated with peripheral vascular lesions. (7) It was also of value in the treatment of infections of the biliary tract. (8) Good results were obtained with aureomycin in 23 of 29 patients who had failed to respond to penicillin. (9) One patient with gonococcal salpingo-oophoritis and one with regional ileitis failed to respond to aureomycin.

In a note added after the paper had been written the authors record that severe proctitis and colitis developed in a number of patients during oral aureomycin therapy. The accompanying diarrhoea was severe enough to impose a grave burden on the patient's fluid and electrolyte balance. *C. G. Rob.*

Terramycin Therapy for Meningitis: a Report of Fourteen Recoveries without Other Medication. HOYNE, A. L., and RIFF, E. R. (1951). *J. Pediat.*, **39**, 151.

The authors point out that before the introduction of the sulphonamides and antibiotics the mortality from meningococcal meningitis was usually 50%, and that from other varieties about 100%. With the sulphonamides the mortality was greatly reduced, but their toxic effects, especially on the kidneys, are a disadvantage. The authors are opposed to intrathecal therapy in any form of meningitis other than [presumably] tuberculous.

They treated 14 cases of meningococcal meningitis in patients aged from 1 to 36 years, of whom 10 had petechiae; in 7 of these petechial smears were positive, and the authors considered lumbar puncture unnecessary. There was laboratory proof in all cases. All patients were given the initial dose of terramycin intravenously (250 mg. for children and 500 mg. for adults, dissolved in 1 ml. per mg. of 5% dextrose). To avoid gastric symptoms the authors suggest that the drug should be given after meals or with a glass of milk. All the patients completely recovered both mentally and physically. In 4 proved cases of influenzal meningitis in patients aged from 6 months to 6 years treatment with terramycin and sulphadiazine resulted in 3 complete recoveries and 1 death (this last patient was desperately ill on admission). One patient with pneumococcal meningitis treated with terramycin alone recovered.

A note at the end of the paper describes the treatment of a further 6 cases of pneumococcal meningitis, with 5 complete recoveries and 1 death, and 3 cases of influenzal meningitis in which the patients recovered.

M. H. Pappworth.

The Treatment of Actinomycosis. (Results of Penicillin Therapy in 15 Cases.) FÖLDVÁRI, F. (1951). *Dermatologica, Basel*, **102**, 77.

Penicillin was given to 15 patients, aged between 13 and 58, who were suffering from actinomycosis; 10 were cured and 5 showed improvement. The cervicofacial region was most commonly affected, and out of 10 such cases 7 were cured. One patient with severe pulmonary involvement was cured, as were 2 of 4 patients with abdominal lesions. In 9 cases actinomycosis was diagnosed on microscopical examination; in 3 cases this examination was negative, but the cutaneous test was positive, while in 2 the results of both the examination and the test were negative. In only 1 of 12 cases was there a positive reaction to the complement-fixation test.

The total dose of penicillin varied from 4,500,000 to 20,000,000 units; either an aqueous or an oily solution was used, both proving equally satisfactory. Secondary infections due to other and sensitive organisms also responded well. One advantage was that this treatment could be given to patients who were very debilitated. There were no complications.

Progress in Antimycotic Chemotherapy. LOPEZ, J. CHABÁS (1951). *Clin. y Lab.*, **52**, 179. Bibliography.

The conditions which should be fulfilled by any effective antimycotic therapy, and the progressive development of agents based mainly on their chemical constitution, are discussed. "D25-novex" (2,2.-dioxi-5,5'-dichlorophenyl sulphide) is accepted as the most active and considered to be superior to the diverse phenols, sulphonamides, or salicylic acid. The toxicity of novex is very low; 6 g. daily in tablets of 0.5 g. produced a blood level of 1.7 to 7.8 mg. per 100 ml. Intramuscular injection in a 10% olive-oil solution is equally effective and, combined with local application, was successful in the treatment of both internal mycosis and cutaneous affections. *E. Vasquez Lopez.*

The Effect of Combinations of Antibiotics on *Pseudomonas aeruginosa* and on *Proteus vulgaris*, *in vitro* and *in vivo*. ARMSTRONG, C. W. J., and LARNER, A. E. (1951). *J. Lab. clin. Med.*, **37**, 584.

Sensitivity tests were carried out on 7 strains of *Pseudomonas aeruginosa* and 4 strains of *Proteus vulgaris* with the antibiotics aureomycin, chloramphenicol, penicillin, dihydrostreptomycin, and terramycin. The antibiotics were used singly in preliminary tests to determine the average minimal bactericidal concentration (M.B.C.) for each drug. In subsequent tests 2 or 3 antibodies were combined in amounts proportional to their relative effectiveness when tested singly.

Certain antibiotics were more lethal to the test organisms when combined than when tested separately. Thus aureomycin and terramycin in combination reduced the M.B.C. for *Ps. aeruginosa* twelvefold, and a mixture of chloramphenicol and terramycin was nine times more effective than either of the drugs used singly. By contrast penicillin and chloramphenicol were antagonistic, and were less effective together than when used separately. The combinations which were most lethal to *P. vulgaris* were aureomycin–streptomycin and streptomycin–terramycin.

Experiments were carried out *in vivo* on mice infected with cultures of the same test organisms. The antibodies were given, singly or in combination, by intraperitoneal injection at the time of the infection and again 6 hours later. The survival rate among mice infected with *Ps. aeruginosa* and treated with aureomycin and terramycin together was 100%, while only 10% survived when treated with the drugs separately. Similar results were obtained with a mixture of chloramphenicol and terramycin. The optimum combination for the treatment of *P. vulgaris* infection was one which included streptomycin, although this drug was relatively ineffective by itself *in vitro*. Streptomycin combined with aureomycin or terramycin reduced the mortality rate among infected mice to 10% and 20% respectively. When the three drugs were used singly the mortality rates fell within the range of 60 to 100%. *G. B. Forbes.*

Steatorrhea following the Use of Antibiotics. MERLISS, R. R., and HOFFMAN, A. (1951). *New Engl. J. Med.*, **245**, 328.

Details are given of 4 patients who had been treated with chloramphenicol, aureomycin, terramycin, or penicillin with sulphonamides for a variety of reasons. About a week after the initial condition had cleared up these patients developed a troublesome diarrhoea which had the character of a mild sprue syndrome. On intensive investigation no causative agent was found, but the diarrhoea was regarded as due to a dysfunction of the small intestine associated with fatty stools. After the patients had lost weight and endured some discomfort success was obtained from the administration of a low-fat diet, crude vitamin-B preparations, and parenteral liver extract.

The authors contend that the syndrome was due to the effects on the flora of the small gut of the oral antibiotics or the penicillin-sulphonamide combination, causing a deficiency state which resembles that seen in sprue. The presence of monilia is considered to be secondary and not of aetiological significance. *James D. P. Graham.*

BIOCHEMISTRY

On Chemical Tests for Blood in Urine. CAPLAN, H., and DISCOMBE, G. (1951). *Brit. med. J.*, **2**, 774.

The absolute and relative sensitivity of microscopical, spectroscopic, and chemical procedures for the detection of blood in urine are described. Microscopical examination (in a Fuchs–Rosenthal haemocytometer) of a deposit, obtained by centrifuging 5 ml. of urine at 3,000 revolutions per minute (r.p.m.), removing 4 ml. of supernatant fluid, and mixing the residue, was found to be capable of detecting 5,000 erythrocytes per ml. of urine; and this sensitivity could be increased about 5 times if the Addis technique (12.5 ml. of urine at 3,000 r.p.m., remove 12 ml., and suspend in the residual 0.5 ml.) was employed. Examination of a 2.5-cm. layer of urine by a hand spectroscope for the absorption bands of oxyhaemoglobin could detect 2.5×10^6 erythrocytes per ml., and the conversion of the haemoglobin to pyridine haemochromogen rendered the test 5 times more sensitive. In the laboratory the authors consider it preferable to examine a centrifuged deposit, and suggest that spectroscopy be used to obtain information on the degree and persistence of haematuria.

The sensitivities of the guaiac, amidopyrine, orthotolidine, benzidine, and reduced phenolphthalein (Kastle–Meyer) tests were compared. The first 2 were of comparable sensitivity to spectroscopy; the most sensitive reaction was found to be that with *o*-tolidine (as described by Zwarenstein, *J. clin. Path.*, 1949, **2**, 145), which could detect 5×10^4 erythrocytes per ml. of urine. The benzidine test was found to be 10 times less sensitive and false positives occurred even with apparently clean tubes. In addition, the presence of an average normal concentration of ascorbic acid in the urine (2 mg. per 100 ml.) reduced the sensitivity of the benzidine test about 5 times,

whereas it had no effect on the *o*-tolidine reaction. The reduced phenolphthalein reagent detected 5×10^8 erythrocytes per ml. of urine but proved troublesome to prepare, although it was the only chemical test that did not give false positives with iodides. The *o*-tolidine also gave false positives with bromides. The authors suggested that *o*-tolidine is the most satisfactory test for blood in urine for ward use and in domiciliary work.

M. J. H. Smith.

The Detection of Occult Blood in Feces Including Observations on the Ingestion of Iron and Whole Blood. PERANIO, A., and BRUGER, M. (1951). *J. Lab. clin. Med.*, **38**, 433. Bibliography.

In view of the increasing importance of the detection of blood in the stools, in relation to cancer of the gastro-intestinal tract, the sensitivity of 4 reagents for the detection of occult blood was compared. Normal subjects on a diet containing no meat or fish needed to ingest the following amounts of blood to give a positive test: with orthotolidine, 1 ml.; with benzidine, 3.5 ml.; with phenolphthalein, 3.5 ml.; and with guaiac, 20 ml. (on unrestricted diets 2 to 3 ml.). The stools of normal persons on unrestricted diets gave positive tests with the first 3 reagents; these were converted to negative reactions within 72 hours on a meat-and-fish-free diet. Ferrous sulphate and chlorophyll by mouth did not cause false positive reactions, nor did slight gum bleeding after brushing the teeth.

In testing urine for occult blood, the inhibitory effect of urine on these reagents was confirmed, the benzidine test sometimes being negative even in the presence of 30 to 40 erythrocytes per high-power field.

It is concluded that orthotolidine is probably too sensitive a reagent, and can easily give misleading results. In practice, patients should be put on a flesh-free diet for 72 hours before testing stools for occult blood by orthotolidine, benzidine, or phenolphthalein; the less sensitive guaiac reagent requires no preliminary restriction.

Peter Story.

Use of N-Acetyl-4-aminoantipyrine (NAAP) in Measurement of Total Body Water. BRODIE, B. B., BERGER, E. Y., AXELROD, J., DUNNING, M. F., POROSOWSKA, Y., and STEELE, J. M. (1951). *Proc. Soc. exp. Biol., N.Y.*, **77**, 794.

The use of N-acetyl 4-aminoantipyrine (NAAP) in place of antipyrine for the estimation of total body water was investigated. After the intravenous injection of 1 g. of N-acetyl 4-aminoantipyrine in normal subjects, the agent is uniformly distributed in all the body fluids in proportion to their water content within 3 hours. In this time, less than 15 mg. is metabolized, and less than 3% bound to plasma proteins. About 92% is excreted in the urine in 48 hours and the plasma concentration falls about 8% per hour. In patients with oedema, pleural effusion, or ascites, equilibration of NAAP in the plasma and fluid concentrations is too slow for the method to be useful.

Methods for estimation of NAAP in plasma and urine are described in detail. The distribution of antipyrine and of NAAP in normal human subjects was compared; the results gave good agreement. It is concluded that NAAP has certain advantages over antipyrine: it is less bound to plasma protein; total body water can be calculated from a single sample of plasma and of urine; as NAAP is estimated colorimetrically the need of an ultra-violet spectrophotometer is obviated.

M. Lubran.

The Role of Resins in the Treatment of Water Retention associated with Cirrhosis of the Liver. MOSER, R. H., ROSENAK, B. D., PICKETT, R. D., and FISCH, C. (1951). *Gastroenterology*, **19**, 336.

The authors, who subscribe to the theory that sodium retention may be one of the factors in the aetiology of the disturbed water balance in cirrhosis of liver, studied the effects of sodium-removing resins in 8 patients suffering from this disease. Clinical and laboratory details of these cases are well tabulated, and the authors were able to control ascites and/or oedema in all. They therefore, while emphasizing the part played by the kidneys in electrolyte metabolism, believe that, if cautiously used, the sodium-removing resins have a definite place in the treatment of cirrhosis of liver.

S. Karani.

Iron Metabolism in Pregnancy. The Behaviour of Haemoglobin, Serum Iron, the Iron-binding Capacity of Serum Proteins, Serum Copper and Free Erythrocyte Protoporphyrin in Normal Pregnancy. VENTURA, S., and KLOPPER, A. (1951). *J. Obstet. Gynaec. Brit. Emp.*, **58**, 173.

Differences of opinion exist between Scandinavian and American workers as to the behaviour of serum iron in pregnancy. This problem was therefore reinvestigated by the authors in 25 non-pregnant controls and in 105 pregnant women divided as evenly as possible between the four 10-week periods of pregnancy and 25 puerperal patients. In addition to serum iron values, those of serum copper, serum iron-binding capacity, and erythrocyte free-protoporphyrin were estimated, the first three by standard methods (with certain modifications) and the last by the method of Grinstin and Wintrobe (*J. biol. Chem.*, 1948, **172**, 459).

Blood for the analyses was taken under standard conditions from fasting patients at about 6 a.m., most of the subjects being admitted to hospital for the purpose. Haemoglobin levels were measured, and cases in which the value obtained was less than 10 g. per 100 ml. blood were excluded from the series. Similarly cases of sepsis, toxaemia, and ante-partum or post-partum haemorrhage were omitted.

It was found that serum iron levels start to fall after the twentieth week of pregnancy and continue to do so until the puerperium, when the fall is halted and the beginning of a return to normal is seen.

Both unsaturated and total iron-binding capacity increase after the twentieth week of pregnancy, the former from 218 μg. per 100 ml. serum in the non-pregnant to 383 μg. per 100 ml. serum in later pregnancy.

In confirmation of the findings of earlier workers, the authors found that serum copper levels progressively rise from 10 weeks onwards, reaching twice the non-pregnant value at term.

As would be expected, the fall in serum iron level is accompanied by an increase in the free-protoporphyrin content of erythrocytes.

Using the figures for plasma volume in pregnancy given by White and by Thomson *et al.*, the authors calculated that in the pregnant woman the total haemoglobin content does not differ greatly from that in non-pregnant women. On the other hand, it would appear that in later pregnancy the decrease in serum iron content is absolute, and not due to the hydraemia of pregnancy. *A. Tickner.*

HAEMATOLOGY

The Antithrombin Titer in Cystic Fibrosis of the Pancreas. A Preliminary Report. INNERFIELD, I., ANGRIST, A., and BENJAMIN, J. W. (1951). *J. Pediat.*, **39**, 287.

The plasma antithrombin was determined in 102 control subjects and in 13 patients with proved cystic fibrosis of the pancreas. Commercial topical thrombin was standardized, using a modification of the method of Quick (*Amer. J. Physiol.*, 1938, **123**, 712). For the test the subject's defibrinated plasma was incubated with a standard thrombin solution. Fibrin was subsequently furnished by the addition of normal plasma at fixed intervals and a clotting time estimated. The speed with which the defibrinated plasma neutralized a known quantity of thrombin was taken as a measure of antithrombin activity. The authors state that antithrombin titres represent a highly sensitive plasma response to the formation of trypsin in the pancreas.

In none of the control subjects (healthy children and infants with chronic diarrhoea, idiopathic steatorrhoea, or marasmus) was the plasma antithrombin titre elevated or diminished. Of the patients with cystic fibrosis of the pancreas the titre was exceedingly low in a 3-week-old infant dying of the disease; it was strikingly high in 3 infants less than 1 year old and normal in 9 patients aged between 2 and 7 years.

On the basis of these findings the authors suggest that the natural course of the disease can be divided into 3 stages. The first, occupying the first few days of life, is of complete or incomplete pancreatic-duct obstruction with pancreatic achylia and a variable amount of parenchymal damage. In survivors a second stage develops, characterized by interstitial pancreatitis secondary to extravasation of obstructed pancreatic enzymes. A third stage—the stage of pancreatic contraction and scarring—follows and is characterized by extensive parenchymal replacement fibrosis, areas of hyaline degeneration, the appearance of corpora amylacea, and the development of large cysts. *Harold Caplan.*

Megaloblastic Anaemia of Pregnancy and the Puerperium. THOMPSON, R. B., and UNGLEY, C. C. (1951). *Quart. J. Med.*, **20**, 187.

Over a period of 17 years 45 cases of megaloblastic anaemia of pregnancy were observed at the Royal Victoria Infirmary, Newcastle-upon-Tyne; 7 of the patients were seen during pregnancy, the remainder having already been delivered before admission to the medical clinic. The mean age of 31 years was considerably lower than that of 55 years found in a random sample of 45 women with Addisonian anaemia. In a comparison with 1,000 consecutive births in Newcastle and with those reported from the rest of the country, the distribution of cases suggested that this disorder tends to occur more frequently in multiple pregnancies than in the first or second pregnancy. One of the women suffered twice from the disease, so that there were altogether 46 cases. Whereas the expected incidence of twins is 1 in 80 births, twin pregnancies occurred 4 times. There were 47 live births and 3 stillbirths. Two infants died shortly after birth; 3 died later, 1 at 3 days (cause unknown), 1 at 3 weeks of cystic cerebral degeneration, and 1 of pyloric stenosis. Megaloblastic bone marrow, indistinguishable from that of Addisonian pernicious anaemia, was present in all the 30 cases in which sternal puncture was performed. Otherwise the haematological picture was not uniform. None of the expected features was seen constantly. Of 38 cases, 10 had a mean corpuscular volume below 90 c.μ; and in 19 cases the mean corpuscular haemoglobin concentration was below 30% and in 5 cases it was less than 25%. In 3 out of 46 cases the mean corpuscular haemoglobin was below 25 μμg. Of 29 cases 21 showed a normal serum bilirubin concentration (less than 0.8 mg. per 100 ml.), whereas in 29 cases of Addisonian pernicious anaemia 25 had an abnormally high serum bilirubin level. The survival rate of transfused normal erythrocytes was followed in 3 cases and there was evidence of increased destruction. This increased elimination of erythrocytes ceased with folic acid and raw liver therapy. Vitamin B_{12} had no effect. Free gastric acid was present in 32 of 41 cases investigated. A dietary history was taken in 27 cases. In only 11 cases could the anaemia be explained on a deficiency basis.

In contrast to cases of pernicious anaemia and of megaloblastic anaemia of pregnancy in India (Patel and Kocher, *Brit. med. J.*, 1950, **1**, 924), the patients in this series did not on the whole respond to anti-pernicious anaemia principle, either in its pure form as vitamin B_{12} or as parenteral liver extract. Folic acid treatment had a rapid therapeutic effect, as had raw-liver pulp and yeast. Whereas beef liver contains 10 μg. of folic acid per gramme and its effect might therefore have been due to folic acid, the response to yeasts suggests the presence of another haematopoietic factor. [It is noteworthy that Wills, who worked on pregnant women in India, isolated the "Wills factor" from "marmite," made from yeast.] *H. Lehmann.*

Trypsin-modified Erythrocytes: their Use as Test Cells in Acquired Hemolytic Anemia. ROSENTHAL, M. C., DAMESHEK, W., and BURKHARDT, R. (1951). *Amer. J. clin. Path.*, **21**, 635.

The authors describe their method of using trypsin-modified erythrocytes for the recognition of abnormal agglutinins in acquired haemolytic anaemia. The patients' erythrocytes and normal compatible erythrocytes were used at temperatures of 37°, 22°, and 3° C. Trypsin-treated erythrocytes in saline agglutinate less readily than erythrocytes in albumin at 37° C., equally well at 22° C., and better at 3° C. On the whole the authors favour the use of trypsin-treated erythrocytes in saline rather than erythrocytes in albumin.

Janet Vaughan.

Experimental Confirmation of a Previously Reported Unusual Finding in the Blood of Cyclotron Workers. INGRAM, M., and BARNES, S. W. (1951). *Science*, **113**, 32.

The authors have previously reported the appearance of lymphocytes with double bilobed nuclei in the blood of subjects associated with the cyclotron at the University of Rochester after exposure below accepted tolerance levels. The experiments in 3 dogs which are recorded in the present paper confirm these findings.

Daily blood studies were carried out for 2 months before the experiment, and only one blood smear was found to have one or more lymphocytes with bilobed nuclei. No other blood abnormalities were found. The dogs were then fastened 50 feet (15.2 m.) from the target and well outside the neutron beam for 30 minutes while the cyclotron was operating. Each dog was exposed once in each of three positions. Although the radiation received was not measured it was believed that the exposures were well below the currently accepted tolerance levels. Approximately 180 blood smears were examined during the first week after exposure and then 30 smears each week. The experiment was continued for one year. Lymphocytes with bilobed nuclei were found in about 20% of the smears during the first and second weeks, and in a diminishing number during the succeeding weeks. After the sixth week the characteristic cells were not found.

Although bilobed lymphocytes are not diagnostic of radiation effects, it is important to note that these changes in the blood were produced by very small exposures. Such changes may suggest that slight exposure is occurring and may indicate that a particular operation is potentially dangerous before more obvious changes appear in the blood of the subjects involved.

T. M. Pollock.

Streptomyces Fermentation Derivatives in Acute Leukaemia. BARNARD, R. D. (1951). *Lancet*, **1**, 1157.

Since 1949 the author has been treating cases of acute leukaemia in the United States with a crude *Streptomyces griseus* fermentation by-product sold as a pig and poultry food supplement. It appears that 50 patients have received this treatment. In some cases the clinical condition underwent considerable, although temporary, improvement, but the blood picture did not show corresponding changes. The substance is rich in vitamin B_{12} but evidence is advanced for the view that its effect in leukaemia is due to its antibiotic activity, which alters the intestinal flora, suppressing the *Bacterium coli-aerogenes*. It is suggested that clinical remissions tend to persist while this effect on the intestinal flora is maintained, and that relapses are correlated with the emergence of antibiotic-resistant strains of microbes. It is also claimed that this event can be postponed by a liberal milk regimen promoting the growth of lactobacilli.

L. J. Davis.

Presence of Foetal Haemoglobin in Cooley's Anaemia. LIQUORI, A. M. (1951). *Nature, Lond.*, **167**, 950.

Crystallized oxyhaemoglobin from a subject with Cooley's anaemia is denatured by alkali more slowly than normal haemoglobin, but faster than foetal haemoglobin: it appears to be a mixture of about equal parts of foetal and normal haemoglobin. The solubility of reduced and oxyhaemoglobin and the fine structure of the tryptophan band near 2,900 A are in agreement with this hypothesis. It is suggested that Cooley's anaemia is due to a congenital inability to complete the change-over from the foetal to the adult form of haemoglobin synthesis.

George Discombe.

The Bone Marrow in Hyperthyroidism and Hypothyroidism. AXELROD, A. R., and BERMAN, L. (1951). *Blood*, **6**, 436. Bibliography.

The author reviews the relevant literature and reports an investigation of the bone marrow in 26 patients with hyperthyroidism and 9 with myxoedema. Sternal marrow was obtained by aspiration biopsy and serial sections were available for study (the examination of serial sections was found to give more reliable information than the volumetric determination of fat and cell content of aspirated marrow samples). In hyperthyroidism there was an increase in myeloid and erythroid cells in the sternal marrow. There was also an increase in lymphoid tissue, and the fat content of the marrow was low. In hypothyroidism the sternal marrow was hypoplastic.

P. C. Reynell.

Demonstration of a Thrombocytopenic Factor in the Blood of Patients with Thrombocytopenic Purpura. HARRINGTON, W. J., MINNICH, V., HOLLINGSWORTH, J. W., and MOORE, C. V. (1951). *J. Lab. clin. Med.*, **38**, 1.

The aetiology of idiopathic thrombocytopenic purpura has been thought to depend upon either a depressed megakaryocyte activity or an increased platelet lysis. The present investigation suggests a further possible cause of the condition—the presence of a thrombocytopenic factor in the blood of such patients. The blood from 8 to 10 patients with this condition produced a profound reduction in the number of circulating platelets when transfused in volumes of 250 to 500 ml. into normal

volunteers. The decrease was of the order of 50% of the initial count, was maximal 2 to 3 hours after injection, was maintained for 5 to 7 days, and was often accompanied by haemorrhagic symptoms similar to those of thrombocytopenic purpura. Transfusions from control subjects had no such effect.

In patients who underwent splenectomy the thrombocytopenic factor was persistent, even though the platelet count had improved. Initial studies suggest that the active factor is contained in the globulin fraction of plasma—a fact of interest in view of the finding of Evans *et al.* (*Arch. intern. Med.*, 1951, **87**, 48) of platelet agglutinins in the plasma of patients with idiopathic thrombocytopenic purpura or that caused by sedormid sensitivity. *H. Payling Wright.*

The Vitamin B_{12} Group of Factors. SMITH, E. LESTER (1951). *Brit. med. J.*, **1**, 151.

This is a short review of recent progress in the vitamin-B_{12} field. Vitamin B_{12a} appears to be identical with vitamin B_{12b}, and to be a reduction product of vitamin B_{12}. The presence of a cyanide group has been established in the vitamin-B_{12} molecule, co-ordinated with the cobalt atom. Vitamin B_{12b} lacks this cyanide group. Two new factors, vitamin B_{12c} and vitamin B_{12d}, have been isolated from *Streptomyces griseus* fermentation liquors. Vitamin B_{12c} can be distinguished from the other factors by its characteristic absorption spectrum. Vitamin B_{12d} and vitamin B_{12b} have identical absorption spectra, but they can be separated chromatographically. The behaviour of vitamin B_{12c} and B_{12d} on microbiological culture plates differs from that of vitamins B_{12} and B_{12b}. With regard to its biological significance there now seems little doubt that vitamin B_{12} is Castle's "extrinsic factor." It has also recently been shown to have a number of other biological activities which are briefly referred to in this article. *L. J. Davis.*

MORBID ANATOMY AND HISTOLOGY

Mesothelioma of the Female Genital Tract. Review of the Literature and Report of Five Cases involving the Uterus. HORN, R. C., and LEWIS, G. C. (1951). *Amer. J. clin. Path.*, **21**, 251.

Five tumours of the female genital tract involving the uterus are described from the Hospital of the University of Pennsylvania. The literature is reviewed, where 24 further cases are recorded; of the total of 29 tumours 13 involved the uterus, 11 were tubal, and 5 ovarian.

The pathological appearance of these tumours varied. Most of the lesions were solid and resembled fibroids. Definite capsules were not always found, but the tumours were generally referred to as circumscribed. Only 4 tumours were cystic; all of these were ovarian. Microscopically, the tumours varied from a net-like structure, displacing normal tissue, to groups of cells scattered through a fibro-muscular background. The cells were either flattened, endothelium-like elements, or cuboidal or even columnar; they were arranged in solid cords through a fibro-muscular background. The cells were frequently vacuolated, containing material resembling mucin. Lymphocytic aggregations were a striking feature of many mesotheliomata.

In 4 of the 5 cases the finding of a tumour was incidental; in the fifth case a tumour believed to be fibroid was removed and proved to be a mesothelioma. It is considered that these tumours present no characteristic clinical features.

The authors state that with rare exceptions mesotheliomata of the female genital tract may be expected to behave as benign tumours; they also consider that this group of tumours may originate from the mesothelial lining cells of the peritoneum. *C. J. Dewhurst.*

Benign Mesotheliomas (Adenomatoid Tumors) of the Genital Tract. LEE, M. J., DOCKERTY, M. B., THOMPSON, G. J., and WAUGH, J. M. (1950). *Surg. Gynec. Obstet.*, **91**, 221.

A rare and interesting neoplasm occurs in the epididymis, spermatic cord, and testicular tunics of the male, and in the uterus, Fallopian tubes, and ovary of the female genital tract. It has been variously designated in the literature, which is here reviewed, but the authors consider it to be a mesothelioma.

This article presents a clinico-pathological study of 35 cases of mesothelioma of the genital tract from the records of the Mayo Clinic.

There were 18 mesotheliomata in the epididymis, 1 in the tunica albuginea, 1 in the tunica vaginalis, 12 in the uterus, 2 in the Fallopian tube, and 1 in the ovary.

Evidence regarding the histogenesis of these unique neoplasms remains inconclusive. Certain microscopical features favour each of the possible sources of origin—that is, epithelium, endothelium, and mesothelium. The authors favour the mesothelial origin.

Subsequent history—as long as 30 years after operation in 1 case in this series—has never revealed local recurrence or metastasis.

Self-healing Epithelioma of the Skin. CHARTERIS, A. A. (1951). *Amer. J. Roentgenol.*, **65**, 459.

This report from the Western Infirmary, Glasgow, deals with a remarkable example of familial incidence of multiple, self-healing, squamous carcinomata of the skin. The father, a man of 69, had suffered from skin tumours since the age of 23. These occurred on the face and forearms, and all regressed in time. He appeared in 1943 with a well-differentiated squamous carcinoma of the perineum which was excised: a large lesion of the forearm was watched for 6 months, during which time it advanced in one area while healing in another, and finally it healed entirely. Histologically, it showed an invasive squamous tumour of high differentiation, with coalescence of large cell nests to form keratin-filled crypts in some areas. Two years later, in 1945, this patient died of rapid and extensive recurrence of the anal tumour. One son, aged 21, had had lesions, some excised, some self-healing, of knees, face, and ears. Another son, aged 38, had lesions of the face, ear, wrist,

and leg. A third son, aged 29, had numerous lesions of face and ears, with a duration possibly of 16 years. Two other sons were not affected: the grandfather probably was affected. It was found that the lesions were much less radiosensitive than might be expected from their histological nature. *B. Lennox.*

My Conception of Cellular Nevi. MASSON, P. (1951). *Cancer*, **4**, 9.

This outline of the neurogenic hypothesis of the origin of pigmented naevi by its originator is clear and timely. The origin of naevi is said to be twofold, from epidermal melanoblasts which multiply and spread into the dermis, and from the Schwann cells of the cutaneous nerves which multiply and infiltrate the deeper dermis. These two components merge and become indistinguishable from each other. The epidermal melanoblasts are specific cells, distinct from the rest of the epidermal cells; their precise source developmentally is still debatable, possibly from the neural crest, possibly from the epidermis itself. In a pigmented naevus which has become malignant it is very difficult to determine histologically whether both of the two components of the naevus may have been the source of the growth. While there is no doubt that malignant change often involves the epidermal melanoblasts, two specimens lead the author to believe that the "neuroid" elements could have been the source of the growths.

R. A. Willis.

Frequency and Form of Primary Cancer of the Liver. ZEITLHOFER, J. (1951). *Krebsarzt*, **6**, 154.

In this paper is reported a study of the incidence of primary cancer of the liver based upon the post-mortem records of the Pathological Anatomical Institute in Vienna. The work was undertaken to investigate whether the use of "butter yellow" has affected the incidence of this disease. The investigation covered three periods: 1900–12, when no butter yellow was in use; 1913–32, when it was used to some extent; and 1938–50, when it was used to a much greater extent. From an analysis of the figures it appears that there has not been any actual increase in primary liver cancer during the half-century, but that the diffuse form of the disease has tended to increase and the nodular form to decrease. When the incidence of cirrhosis of the liver was investigated, there was found to be a significant increase over the same period. [Similar figures for large series of cases are required to exclude the errors which are inevitable when dealing with small numbers in a disease of such rarity.] *H. Russell (Excerpta Medica).*

Primary Carcinoma of the Liver in Hemochromatosis. WARREN, S., and DRAKE, W. L. (1951). *Amer. J. Path.*, **27**, 573.

The incidence of hepatic carcinoma in various reported series of post-mortem examinations in cases of haemochromatosis ranges between 11% and 43%. In a personal series of 20 cases 6 cases of carcinoma of the liver were found—an incidence of 30%. Combining the authors' figures with those of earlier reports, among 127 cases of haemochromatosis there were 24 of hepatic carcinoma—an incidence of 18.9%. The average age of the patients in the authors' cases of uncomplicated haemochromatosis was 56.3 years, and of those with carcinoma 65.5 years. Carcinoma occurred in some cases with only mild cirrhosis, and cannot be attributed solely to excessive regenerative hyperplasia resulting from cirrhosis. The frequent development of carcinoma in haemochromatosis "is doubtless linked to the cause or causes" of this disorder, which "provides a setting for carcinogenesis more effective than simple portal cirrhosis."

R. A. Willis.

Glycogen Storage Disease. I. Familial Cardiac Glycogen Storage Disease: Report of Two Cases and Discussion of Relation to Other Forms of Abnormal Glycogen Deposition. LANDING, B. H., and BANGLE, R. (1950). *Bull. int. Ass. med. Mus.*, **31**, 84. Bibliography.

The clinical features and necropsy and histological findings are described in 2 cases of cardiac glycogen-storage disease occurring in brothers who died aged 43 months and 2¼ months repectively. Necropsy was performed 5 and 8 hours after death respectively and the chief finding was gross cardiac enlargement. The heart weights were 67 g. (normal 27 g.) and 88.5 g. (normal 23 g.). The ductus arteriosus was closed and there was a patent, slit-like foramen ovale in both cases. Histologically, the heart muscle fibres contained large central "vacuoles" surrounded by a fringe of myofibrils. The "vacuoles" failed to take fat stains, but were coloured by Best's carmine and periodic acid-leuko-fuchsin stains.

On searching the literature the authors found 35 cases of the disease reported, of which 31 are summarized in tabular form. The condition appears to occur in two well-defined age groups, namely, in infancy and during adolescence, and, like many other metabolic disorders, is the more severe and rapidly progressive, the younger the patient. From the literature available the authors conclude that cardiac glycogen-storage disease differs from the hepatic form in that the former may be a sex-linked condition, whereas the latter probably is not. The available evidence is inconclusive as to whether the disease is inherited as a Mendelial dominant or recessive character. No case embodying both cardiac and hepatic glycogen storage has so far been described.

Single and multiple rhabdomyomatosis is probably commoner than cardiac glycogen-storage disease, the histology of the two diseases is different, and the former condition tends to be associated with tuberous sclerosis.

"Diffuse cardiac rhabdomyomatosis" in all probability does not exist as a definite disease entity.

R. B. T. Baldwin.

Correction:—Professor Ghalioungui regrets that he omitted to include the name of Professor M. M. Fikry in the acknowledgments to his paper "The Cholesterol Tolerance Test in Normal Individuals" (*J. clin. Path.*, **5**, 203).

J. clin. Path. (1952), **5**, 305.

THE RUNNING OF STANDARDS IN CLINICAL CHEMISTRY AND THE USE OF THE CONTROL CHART

BY

RICHARD J. HENRY AND MILTON SEGALOVE

From the Bio-Science Laboratories, Beverly Hills, California

(RECEIVED FOR PUBLICATION JANUARY 4, 1952)

The progress of clinical medicine has been considerably aided in the past few decades by the increased number of laboratory tests available and the improvement in their specificity and accuracy. Nevertheless, the reliability of analytical results from clinical laboratories has been questioned by many individuals, and several independent surveys have more than justified the suspicion (Belk and Sunderman, 1947 ; Shuey and Cebel, 1949).

In these surveys blame has been placed on poor supervision of personnel, poorly trained and insufficient personnel, poor equipment, poor choice of methods available, and so on. The vast majority of clinical chemical analyses are and probably will continue to be performed by, and supervised by, people not trained as chemists. In such cases laboratory staff follow methods in the so-called "cook-book" fashion with only sparse knowledge of the chemistry involved. Faced with this problem, it becomes the job of the clinical chemist to outline procedures in detail so as to make them as fool-proof as possible.

It is the purpose of this paper to discuss the importance of running routine standards with clinical chemical analyses, the types of standards that can be employed, and the treatment of the data accumulated so as to give reasonable assurance that the results being obtained are within a known permissible error. By such means the laboratory can strengthen the reliability of its results and the faith of the physician in them.

The Use of Standards

Photometric analyses make up the bulk of quantitative clinical chemical analyses. The chemist considers the running of frequent standards with photometric or spectrophotometric analyses routine, but in the clinical laboratory this has seldom been true since the advent of the photocolorimeter. "Precalibrated" photometers are widely advertised to-day with the implication that the tests need not be standardized by the buyer. The acceptance of such precalibrations cannot be too severely condemned. By relying on precalibrations errors can occur as a result of making or buying new reagents, the deterioration or contamination of old reagents, the use of incorrect light filters, changes in the characteristics of the photometer itself, etc. Standardizing each test when a new instrument is obtained is not enough, since the same errors can and do develop subsequent to the standardization. Assuming the necessity of running standards, there is the question of how frequently they should be run. Ideally they should be run with every set of determinations, or once a day (Archibald, 1950). Some have advocated running them twice a week (Levey and Jennings, 1950). Certainly this is far better than not running them at all, but a definite risk is being taken in the latter case, because several days may elapse before serious error is detected. The authors have seen a refrigerated biuret reagent deteriorate within 48 hours to the extent that standards read 30% below what they should have read. Whether this risk is justified is a question which is answered by the individual situation and from experience gained with a particular test over a period of time.

Types of Standards

The Pure Standard.—This is the usually recommended standard and consists of a solution of the substance of known purity with perhaps a preservative added (e.g., benzoic acid in the case of glucose standards). Any other type of standard must first be standardized against a pure standard. The advantages of using this type of standard are that the purity is usually known with a high degree of accuracy, and, with a few exceptions, such as enzymes standards, such standards

are readily available. The disadvantages include (1) the danger of prejudicial handling, since the person running the test knows beforehand what the results should be; (2) substances normally present in blood which may interfere with or augment the colour are not present; and (3) pure standards usually are not run through the entire procedure. With some exceptions, however, there is no reason why pure standards cannot be run through the whole procedure, and, where possible, it is usually advisable. The danger of omitting certain steps is rather obvious. For example, in the determination of blood glucose the usual way to run a standard is to treat the standard glucose solution as if it were a blood protein-free filtrate. The reagents used in preparing the protein-free filtrates of the unknowns are thus by-passed and are not controlled. Any contamination of these reagents leading to alteration of results in the unknowns would not be impressed on the results of the standard.

Pooled Blood or Serum.—This type of standard has been suggested by Archibald (1950) and by Levey and Jennings (1950). The obvious advantage is that it can be introduced as a routine sample, thus eliminating the possibility of prejudicial handling. Furthermore, it must be run through the entire procedure. The primary disadvantage is that it is not at present easily available to all laboratories. Although it is quite probable that most of the chemical values are stable in blood in the frozen state, relatively little actual data are available. Levey and Jennings found that specimens stored in the deep freeze were stable for urea, total protein, albumin, chloride, and CO_2, but not for globulin.

Internal Standard.—This procedure involves the addition of a known "pure standard" to an aliquot of one of the unknown samples. Such a standard would, of course, have to be run through the entire procedure and is readily available. The internal standard is usually utilized in analytical chemistry when there is the possibility of substances present in the unknown which will interfere with the development of colour in the analysis. To check for such interference in every sample would require running an internal standard on every unknown.

Blanks

The use of blanks on the materials used in the tests is nearly as important as the standard. When pooled blood or serum is used as the standard, contamination of a reagent would result in an elevated value for the standard. A reagent blank, therefore, would not be necessary to indicate that trouble of some nature was present; it would, however, indicate the source of the trouble, and if the contamination were not gross the run might be salvaged. But, with the other types of standards, "pure standards" and "internal standards," reagent blanks may be essential. For example, in the analysis of phosphate by the method of Fiske and Subbarow (1925), the standard usually employed is monopotassium phosphate in trichloracetic acid. The trichloracetic acid used for making the protein-free filtrate of the serum sample, however, is not the same trichloracetic acid present in the standard solution. Contamination of this reagent or the water used in the unknown in conjunction with trichloracetic acid would be missed unless a blank were run. In fact, single-distilled water on occasion can contain significant amounts of phosphate and ammonia.

It is suggested, further, that where the absorption of a reagent blank is low, reagent blanks, standards, and unknowns all should be read versus distilled water set at 100% transmission. In this way they are always read against a blank of fixed optical density.

Definitions of Accuracy, Precision, and Reliability

Before discussing the problems of replication of standards and unknowns and the ways of handling data, it is best to define the terms accuracy, precision, and reliability as commonly used to-day in the statistical approach.

Accuracy of a result consists of comparing the observed result with the actual true value. If a known pure standard is used, the accuracy of a procedure frequently can be estimated by recovery experiments with internal standards. In many cases no true value can be determined and a strict determination of accuracy is not possible.

Precision is the degree of variation in results obtained by a method when the same sample is run repeatedly: in other words, the reproducibility of what is observed. The less variation observed, the greater the precision. Obviously, precision can be determined with a high degree of accuracy for every test procedure.

The reliability of a test is the ability to maintain its accuracy and precision into the future. If a test has maintained a steady state of accuracy and precision over a long period of time, then one can predict these characteristics for the future with assurance and the test is said to be reliable. Reliability of the test (which includes the test, analysts, and equipment) can be established only by checks over a period of time.

Control of Accuracy and Precision

The accuracy of a test is a characteristic occasionally beyond the control of the analyst. Various methods for determining the same substance may give different results due to differences in specificity. Blood glucose determinations are a good example of this, since with some methods, for example that of Folin–Wu, reducing substances other than glucose normally present in blood are determined as glucose. Such a test has poor accuracy but may have good precision. This does not necessarily negate the value of the test, especially if the "interfering" substances do not vary markedly, since the clinician becomes accustomed to the definite set of normal values given by the test.

The control of precision requires more serious consideration. For example, if a report on a blood glucose concentration is 140 mg.%, it is important that the clinicians have reasonable assuredness that the true value by the method used is not 110 or 170 mg.%. "Reasonable assuredness" is commonly defined in statistical analyses as "95% confidence," i.e. the value can be reported with a range limit on each side, within which range the true value, by the method used, will be found 95 times out of 100. More rigorous "confidence limits" can, of course, be used. If it is found for the glucose determination used in our example that the confidence limits are $\pm 10\%$ of the observed value, then the clinician can be given the "reasonable assurance" in the form 140 mg.% $\pm$ 10%, or 140 mg.% $\pm$ 14 mg.%. Ideally each report should be accompanied by some indication of its precision, although routine reporting of clinical chemical determinations with confidence limits is probably impracticable. It is important, however, that laboratories and clinicians become aware of them and think in terms of them.

The precision, i.e. confidence limits, of an analytical procedure can be determined by several methods. The most obvious is to run numerous replicates, say 30, of a sample and analyse mathematically the dispersion of results obtained. Such an analysis will be considered in detail in the subsequent section on control charts. Although this solution of the problem is valid, it is not very practicable in the routine operation of most clinical laboratories. A much simpler approach merely requires running routine unknowns in duplicate at least until about 30 duplicate analyses are accumulated. The duplication must be complete, i.e., through the entire procedure. The difference between each pair, the range R, is calculated and the arithmetic average or mean of the ranges, $\bar{R}$, is calculated. The magnitude of the confidence limit on each side of a *single* observation is then estimated by multiplying $\bar{R}$ by 2.65.*

Table I shows the calculation of the confidence limit in the determination of glucose, using only 10 pairs.

TABLE I

Blood Glucose (mg.%)	R
100, 103	3
86, 94	8
120, 129	9
97, 92	5
126, 125	1
79, 83	4
104, 104	0
120, 108	12
84, 88	4
93, 95	2
Total =	48

$$\bar{R} = \frac{48}{10} = 4{\cdot}8$$

$$\text{Confidence limit} = \pm 2{\cdot}65\,(\bar{R}) = \pm 12{\cdot}7 \text{ mg.\%}$$

The confidence limit as calculated in Table I is in terms of mg.% which, when converted to per cent error by referring it to the approximate average of the range of analyses used (100 mg.%) becomes $\pm$ 12.7%. In neither of the above methods of estimating precision is the error contributed by variation in reagent blanks included in the estimate. This will be dealt with in detail in a future publication, where it will be shown that usually there is only a small increase in the limits as calculated above.

The first decision which must be made before setting up any clinical chemical procedure is the degree of precision required, i.e., how wide dare the confidence limits be. Blood glucose values will probably be satisfactory for clinical use,

* This is an application of the use of range for estimation of standard deviation. A confidence limit of three standard deviations is obtained for the mean of pairs by multiplying R by 1.88. Multiplying this by 1.4 gives the limit for a single observation, i.e. 2.65 (R). Justification for the use of three standard deviation confidence limits is discussed in the section on control charts.

though perhaps not for research, if they are within 10% of the correct value for the method used. Many clinical chemical determinations are satisfactory for clinical use if they have a precision in this range. There are some determinations where an error of this magnitude obviously might be serious. The normal range for serum sodium is approximately 300 to 345 mg. per 100 ml. Thus a value in the middle of this range with confidence limits of $\pm 7\%$ would blanket the entire range. Even for clinical use a result with a maximum error of approximately 3% is thus required. Similarly, a value in the middle of the normal range of serum calcium, if taken as 9 to 11 mg.% with limits of $\pm 10\%$, would include the whole range.

The next problem to consider is what can be done to decrease the magnitude of the confidence limits (or error) of a determination once it is found that the existing limits are too large. In designing the routine procedures of a laboratory one must stay within the bounds of practicability and still be assured of the accuracy and precision desired. Valuable time in the laboratory is wasted by complicating procedures to give greater precision than is actually required. If a procedure, however, lacks the required precision, one obvious line of attack is to use more precise and careful technique in its performance or improve the method itself, e.g., better control of variables, such as temperature, which may affect the result. Another possible approach to decreasing the error is replication, i.e., running the unknown sample in duplicate, triplicate, etc. Statistics must be called upon to answer just how much of an increase in precision is to be expected (Simon, 1941). If E is the observed confidence limit or error for a single determination and $E_{\bar{x}}$ the maximum confidence limit or error desired, then the number of replicates, N, necessary to achieve this is $\left(\frac{E}{E_{\bar{x}}}\right)^2$.

To demonstrate the magnitude of this increase in precision with replication let us presume an error of $\pm 10\%$ with a single sample. The error for various numbers of replicates can be found by substituting in the transposed version of the formula $E_{\bar{x}} = E/\sqrt{N}$. For one, two, three, four, and five replicates the error would be 10, 7, 5.8, 5, and 4.5% respectively. Thus the error decreases inversely as the square root of the number of replications. If the replication necessary is greater than duplicates, or perhaps rarely triplicates, this method of increasing precision becomes totally impracticable in the clinical laboratory. If neither of these approaches yields the required precision or if they are impracticable, the method must be discarded and a search made for a more precise method.

What has been said for unknown samples also holds to a certain extent for standards. Reagent blanks present the same problem, but in a much attenuated form. In the vast majority of cases the light absorption by the reagent blanks is very small compared with that absorbed by the standard and unknown, so that even a rather large relative error in the reagent blank would not often materially alter the correction factor of the blank. It is an entirely different matter if the reagent blank absorption is relatively high. As previously stated, the frequent running of reagent blanks is usually for the purpose of checking the reagents for contamination.

Control Charts

After a test has been set up and standardized and periodic standards run to check on the procedure, the simplest way to keep track of these results is merely to note them in a notebook reserved for the purpose. As expected, the results will vary from day to day, and if one value is suddenly greatly out of line suspicion will be aroused. There is a simple way, however, to treat these check data as they accumulate which has the advantage of telling the analyst at a glance how much the checks can vary before suspicion is warranted and action indicated. The use of the quality control chart will frequently predict trouble before it actually happens (Mitchell, 1947; Wernimont, 1946).

There are numerous ways in which these charts can be set up, but only a few of the simplest will be discussed, because they will serve the purpose admirably in the clinical laboratory.

Type I.—For this type single standards are run. A sheet of graph paper is employed (it is convenient to have a loose-leaf notebook of graph paper for quality control charts of the various determinations) with an appropriate scale on the ordinates for the observed result of the standard. This scale may be in terms of the final result, e.g. mg. %, but it is usually more convenient to use the density reading of the standard (corrected for reagent blank if read versus water or solvent blank). The abscissae represent successive determinations and the date is noted for each point placed on the graph. The next step cannot be taken until 20 such points have been plotted, so it is advisable to run standards frequently, perhaps once a day, at

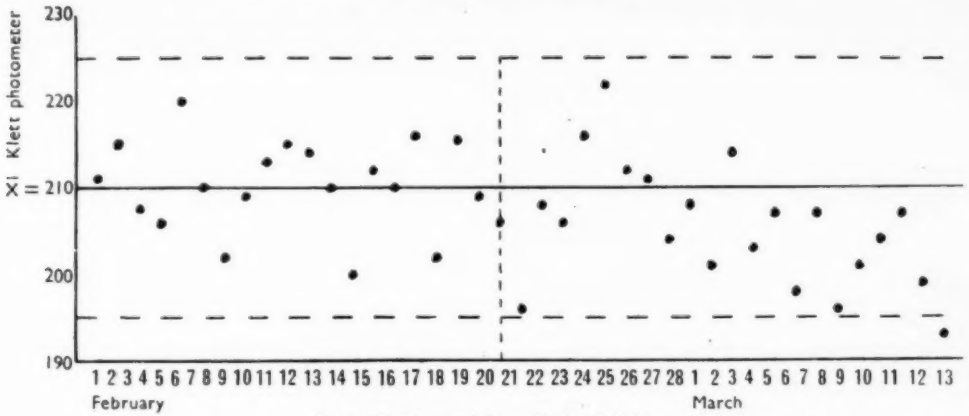


FIG. 1.—Type I control chart. Single standards.

least until this number has been reached. The arithmetic average or mean, $\bar{x}$, is then calculated from these 20 values and represents the best estimate of the true value for the standard. A horizontal line is then drawn on the graph at this point as in Fig. 1.

The next step is to establish confidence limits for the standard values. These are the same confidence limits mentioned before and constitute a range of equal magnitude on both sides of the mean, $\bar{x}$, in which approximately 95 times out of 100 the standard result should fall if *no factors are operating other than those operating during this period in which the 20 values were obtained.* These limits are calculated as follows:

If N = number of determinations

$\bar{x}$ = arithmetic mean of N determinations

Σ = "sum of"

σ = standard deviation

x = a single determination

$(\bar{x}-x)$ = the deviation of a determination from the mean, $\bar{x}$.

$$\bar{x} = \frac{\Sigma(x)}{N}$$

$$\sigma = \sqrt{\frac{\Sigma(\bar{x}-x)^2}{N-1}}$$

Often the mathematical labour is simplified by the use of another form of this equation:

$$\sigma = \sqrt{\frac{\Sigma(x^2) - \frac{(\Sigma x)^2}{N}}{N-1}}$$

As an example the calculations for the confidence limits of Fig. 1 are presented herewith:

TABLE II

(x)	$(\bar{x}-x)$	$(\bar{x}-x)^2$
211	1	1
215	5	25
207	3	9
206	4	16
220	10	100
210	0	0
202	8	64
209	1	1
213	3	9
215	5	25
214	4	16
210	0	0
200	10	100
212	2	4
210	0	0
216	6	36
202	8	64
213	3	9
209	1	1
206	4	16

$\Sigma(x) = 4200$ $\Sigma(\bar{x}-x)^2 = 496$

$N = 20$

$$\bar{x} = \frac{\Sigma(x)}{N} = \frac{4200}{20} = 210$$

$$\sigma = \sqrt{\frac{\Sigma(\bar{x}-x)^2}{N-1}} = \sqrt{\frac{496}{19}} = 5{\cdot}1$$

$3 = 15{\cdot}3$

The confidence limits are drawn above and below the line drawn for the x equal to a distance of 3σ. If all goes well for many more determinations, say a total of 100, the $\bar{x}$ and s can be re-evaluated. It will be noted that if the variation of determinations about their mean represented the so-called "normal" or Gaussian distribution (symmetrical bell-shaped distribution), the 95% confidence limits would be represented by $\pm 2\sigma$. It has been emphasized, however, that distributions of this type are frequently not of the "normal" type (Mitchell, 1947; Clancey, 1947). Clancey (1947) analysed the variation of many chemical analyses and found the Gaussian distribution to hold in a minority of cases.

There are always two dangers when using control charts: (1) looking for trouble that does not exist, and (2) not looking for trouble that does exist. Thus, the use of 3σ as compared to 2σ may reduce the number of times that we look for trouble when it does exist, and may increase the number of times that trouble exists and we do not look for it. Taking these points into consideration, and especially when there is no *a priori* knowledge of the nature of the distribution, it is believed that the best compromise, and certainly the safest, is to associate "reasonable assuredness" with confidence limits of $\pm 3\sigma$ (Simon, 1941; Mitchell, 1947), especially when 3σ limits are within the desired precision and we do not want to waste time looking for trouble that does not exist. If, however, 3σ limits are too broad compared with the precision required, 2σ limits may be preferred if we are willing to spend the time to investigate out of limit points knowing that frequently in such cases trouble does not actually exist.

What should be done if a value falls outside the confidence limits? First of all it must be remembered that these are only "reasonable assuredness" confidence limits, and therefore approximately one value in every 20 to 100 should fall outside, but not far outside. If a value falls slightly outside the limits, the procedure should be checked carefully for an assignable cause—new reagent used, new standard, possible break in technique, etc. If none of these are obvious and the possible error involved, if it is an error, is not critical, you may wait to see what happens next time. If the same thing happens next time, then there is cause for alarm, since there is a good indication that there is trouble. Many times trouble can be predicted before it actually happens. In Fig. 1 it is noted that after February 26 there is a gradual drift in the distribution in a downward direction, although not until March 13 is there a value falling outside the lower confidence limit. Obviously, trouble began brewing about February 26, but not until March 13 can the test be considered out of control. This type of drift occurs when one reagent gradually deteriorates, e.g., aminonaphtholsulphonic acid reagent in the determination of phosphate.

Type II.—In a Type I control chart there is a certain amount of mathematical labour involved in the calculation of σ. This can be obviated if successive determinations are treated as pairs (Wernimont, 1946). The mean of each pair and the differences between each pair (the range, or R) are plotted as in Fig. 2. The 95% confidence limits for the $\bar{x}$ are $\pm$ 1.88 ($\bar{R}$), where the value of $\bar{R}$ is obtained by averaging the range values, R. The confidence limit for the range is 3.27 (R). (Only one limit need be considered, since the other limit is obviously 0.)

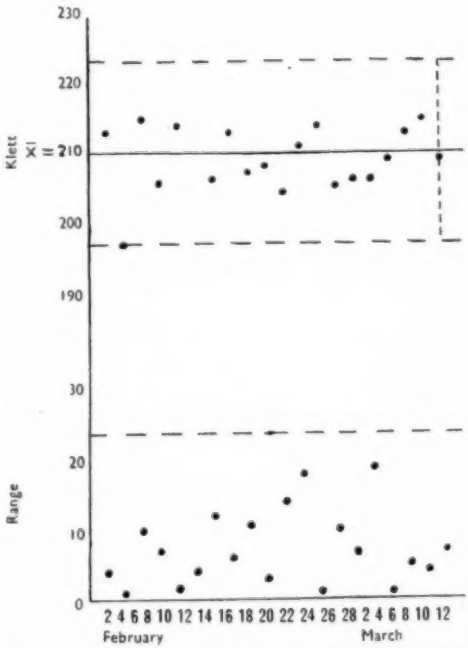


FIG. 2.—Type II control chart. Successive single standards paired.

Type III.—More information can be obtained by using duplicate standards. The difference between the pair of standards or the range, R, is calculated and plotted each time as in Type II and the confidence limits calculated as in Type II. The main advantage of this procedure is that it is possible to tell whether the variation between days is significantly greater than the variation in any one run. With many chemical procedures there is a definite tendency for this to be so, signifying the presence of insufficiently controlled variables, such as temperature. Such a situation would be indicated when the range values are apparently in control but the averages of the duplicates out of control. This type of control chart has been suggested for use in the clinical laboratory by Levey and Jennings (1950), who give some examples of its use and how it detects errors which develop in procedures. Whether the added labour in running duplicate standards is worth while depends on the individual situation.

No matter which type of control chart is used, the value for the standard used in the calculations of the unknowns is the mean $\bar{x}$, not the value for the standard obtained on the same day or even in the same run with the unknowns.

If unknown samples are run singly, the error should be the same as that determined for standards if a Type I control chart is used, *provided the unknowns and standards are treated the same.* Similarly, if unknowns are run in duplicate the error of their average should be the same as that determined for standards run in duplicate (Type III). This assumes that the error is the same for unknowns as for standards and is approximately correct if the per cent transmission for both is in the range of 20 to 60% (optical density 0.2 to 0.7). When pure standards are used and not run through the entire procedure the confidence limits set for the standards obviously cannot be used as an estimate of the precision of unknowns.

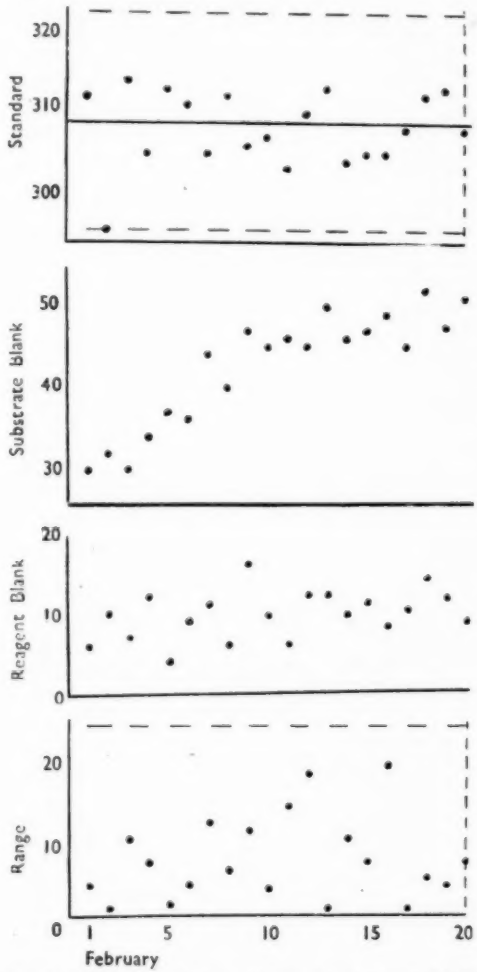


FIG. 3.—Type III control chart with reagent and substrate blanks recorded.

In a procedure such as the determination of serum phosphatase the substrate blank, the reagent blank, and the range for standard, if a Type II or III control chart is being used, can all be plotted together as in Fig. 3.

There is no practical way, and in many cases it is impossible, to be sure that the result of any one determination is correct even with the running of standards and blanks. The procedures outlined here, however, will tell in many cases the accuracy to be expected by a procedure, the precision in all cases, and the reliability as indicated by quality control charts.

There are other factors of importance in setting up determinations which are outside the scope of this paper and have received adequate treatment elsewhere (Archibald, 1950; Ayres, 1949). These include the proper choice of concentration range (yielding final optical densities of 0.2 to 0.7), and the establishment of whether or not the colour produced follows Beer's law.

Summary

It is considered absolutely essential to run frequent standards in all colorimetric clinical laboratory analyses. The various types of standards that can be run are discussed, as well as the estimation and control of accuracy and precision, and the role of duplication in the control of precision. The employment of control charts is advocated, since their use allows the laboratory to be aware at all times of the state of control of the test.

The authors wish to express their appreciation for the advice given by Frank B. Cramer on some of the statistical points.

REFERENCES

Archibald, R. M. (1950). *Analyt. Chem.*, **22**, 639.
Ayres, G. H. (1949). *Ibid.*, **21**, 652.
Belk, W. P., and Sunderman, F. W. (1947). *Amer. J. clin. Path.*, **17**, 853.
Clancey, V. J. (1947). *Nature, Lond.*, **159**, 339.
Fiske, C. H., and Subbarow, Y. (1925). *J. biol. Chem.*, **66**, 375.
Levey, S., and Jennings, E. R. (1950). *Amer. J. clin. Path.*, **20**, 1059.
Mitchell, J. A. (1947). *Analyt. Chem.*, **19**, 961.
Shuey, H. E., and Cebel, J. (1949). *Bull. U.S. Army med. Dep.*, **9**, 799.
Simon, L. E. (1941). *An Engineers' Manual of Statistical Methods.* New York.
Wernimont, G. (1946). *Industr. Engng Chem. Anal. Ed.*, **18**, 587.

J. clin. Path. (1952), **5**, 312.

THE MICRO ESTIMATION OF SERUM IRON AND IRON-BINDING CAPACITY IN NORMALS AND IN DISEASE

BY

G. DAVIES, B. LEVIN, AND V. G. OBERHOLZER

From the Department of Pathology, Queen Elizabeth Hospital for Children, London

(RECEIVED FOR PUBLICATION APRIL 2, 1952)

It is now well established that the transport of iron in plasma is by a specific iron-binding β_1 globulin component of the plasma protein which has been isolated (Schade and Caroline, 1946). Holmberg and Laurell (1945) concluded that the amount of iron in normal plasma by no means attains the limit of saturation of the specific iron-binding protein. This saturation limit has been termed by Laurell (1947) the iron-binding capacity of plasma, and he, as well as Rath and Finch (1949) and Cartwright and Wintrobe (1949), has shown that the simultaneous estimation of serum iron and iron-binding capacity, giving the percentage saturation, may be more informative than the simple determination of serum iron.

Apart from the thiocyanate method of Burch, Lowry, Bessey, and Berson (1948), which requires only 20 c.mm. of serum, most methods for the estimation of serum iron require at least 1 ml. of serum. However, since this investigation was begun a method using a new colorimetric reagent for iron, o-tolidine, has been described (Budtz-Olsen, 1951) requiring only 0.75 ml. serum for duplicate determinations. For the iron-binding capacity, only two methods have been described (Laurell, 1947; Cartwright and Wintrobe, 1949; Rath and Finch, 1949) and both require at least 2 ml. serum. In an investigation of serum iron and iron-binding capacity in infants and the newborn it was felt essential to devise a method suitable for the small amounts of blood which could be obtained by heel or finger prick rather than to limit the scope to those instances where adequate amounts could be obtained by venipuncture. From a review of the literature it appeared that the o-phenanthroline method of Heilmeyer and Plötner (1937), as modified by Laurell (1947), for both serum iron and iron-binding capacity could be most readily adapted as a micro-method. It possessed the advantage that fundamentally the same procedure was used for the two estimations, and that the presence of small amounts of haemoglobin did not introduce any appreciable error.

Experimental

In experiments requiring large amounts of serum these were obtained by venipuncture, observing the usual precautions to avoid contamination with iron. In all other estimations capillary blood from ear or finger was used. All glassware used for the collection of blood or in the analytical procedure was rendered iron-free as described below.

The use of 0.2 ml. amounts of serum instead of 6 ml. as in the original method of Laurell (1947) made it necessary to centrifuge the mixture after the precipitation of protein with trichloracetic acid instead of filtering. The supernatant must be clear, as even the slightest opalescence will, owing to the very faint colour developed, greatly increase the error of the Spekker reading. In the original method the filtrate obtained after removal of the protein with trichloracetic acid is neutralized, first with concentrated ammonium hydroxide solution and then with 0.5 N hydrochloric acid, using p-nitrophenol as indicator. The small volumes of the reagents employed make this neutralization a difficult and time-consuming procedure. Experiment showed that it could be simplified by adding to the supernatant obtained after centrifuging a given amount of concentrated ammonia solution sufficient to reduce the acidity to approximately *p*H 5, followed by a fixed volume of a saturated solution of sodium acetate as buffer. The colour is developed in a solution whose final volume is only 0.5 ml. so that the micro cells of the Spekker absorptiometer must be used, and hence great care must be taken to ensure consistent readings. The small volumes of serum and reagents required were measured with pipettes of the Ostwald–Folin type, made and calibrated in the laboratory.

Estimation of Serum Iron

The serum iron is estimated by liberation with hydrochloric acid, and, after the removal of protein, allowing the iron to react with o-phenanthroline in the presence of sodium hydrosulphite.

Reagents.—The following reagents are required.

6N Hydrochloric Acid.—Constant boiling hydrochloric acid prepared in the usual way in an all-glass apparatus was taken as 6N.

Trichloracetic Acid (20% w/v).—This was prepared from redistilled trichloracetic acid.

12N Ammonium Hydroxide Solution.—This was prepared from 0.880 ammonia solution by dilution with iron-free water. The strength must be checked from time to time.

Phenanthroline Solution.—This solution was of 0.5% w/v in a 10% solution of ethyl alcohol in water.

Sodium Hydrosulphite.—This could only be obtained in the technical grade, and could not be readily purified. All specimens we have been able to obtain, even those said to have less than six parts per million of iron, contain as impurity many small black particles, probably carbon. These contain iron, as is shown by the development of colour when treated with o-phenanthroline, and may give rise to errors due to random sampling. They are removed by sifting through a fine sieve. Since sodium hydrosulphite even in the solid state deteriorates on exposure to air, it was found preferable to keep only a small amount in a separate bottle for immediate use. The addition of the very small quantity of the solid was accomplished by means of a glass capillary scoop calibrated by weighing five combined deliveries. A solution of sodium hydrosulphite may be used, providing it is freshly prepared and stabilized with sodium acetate, in which case 0.01 ml. of a 3% solution in 0.1 N sodium acetate solution is added instead of 0.3 mg. of the solid.

Standard Iron Solutions.—Cleaned pure iron wire (100 mg.) was dissolved in 1.2 ml. of 6N hydrochloric acid, and made up to 1 l. with iron-free water. By appropriate dilutions with redistilled water a range of standard solutions containing from 60 μg. to 260 μg. iron per 100 ml. were obtained.

All glassware was made iron-free by immersion in concentrated hydrochloric acid for two days, then rinsing several times with distilled water, and finally three times with redistilled water. The iron-free water was obtained by redistillation of distilled water in an all-glass apparatus.

Procedure.—To 0.2 ml. of serum in a small test-tube is added drop by drop and with shaking 0.1 ml. of 6N hydrochloric acid. The mixture is allowed to stand for 10 minutes, after which 0.2 ml. of 20% trichloracetic acid solution is added drop by drop and with shaking and allowed to stand a further 10 minutes. After centrifuging at high speed for about 10 minutes 0.37 ml. of the clear supernatant is taken into a 2 in. × ¾ in. rimless test-tube calibrated at 0.55 ml. To this, 0.05 ml. of 12N ammonium hydroxide solution is added, followed by 0.05 ml. of a saturated solution of sodium acetate. Then approximately 0.01 ml. of the phenanthroline solution is added and finally 0.3 mg. of solid sodium hydrosulphite. The reagents are mixed well by tapping after the addition of each. Finally the solution is made up to the calibration mark with redistilled water and again mixed. The full colour develops immediately, and may be read after only a few minutes, or, since the colour is quite stable, after standing for an hour or longer. In this investigation a Spekker absorptiometer and 1-cm. depth micro cells with the spectral green (Ilford No. 604) filter were used, the readings being made against water. When carrying out a batch of serum-iron estimations together a series of standard iron solutions and a reagent blank were always included. The value of the serum iron may be read off directly on the linear curve obtained by plotting the Spekker readings of the standard iron solutions against the iron content (Fig. 1).

Estimation of Total Iron-binding Capacity of Serum

In this method iron is added to serum in an amount more than sufficient to saturate the iron-binding β_1 globulin component. The excess is allowed to react with phenanthroline in the presence of sodium hydrosulphite. The addition of hydrochloric acid liberates the iron from the iron-globulin compound but not from the iron-phenanthroline complex, which is precipitated, together with all the protein, by trichloracetic acid. The liberated iron, representing the total iron-binding capacity of the serum, is then estimated as for serum iron.

Reagents.—The only additional reagent required is a solution containing 50 μg. iron per ml. This is reduced immediately before use by the addition of 20 mg. ascorbic acid to 10 ml. of iron solution.

Procedure.—To 0.2 ml. serum in a small test-tube is added 0.025 ml. of the freshly reduced 50 μg./ml. iron solution. Then 0.01 ml. of phenanthroline solution is added, followed by 0.3 mg. of sodium hydrosulphite. The solution is well mixed by tapping, and, after standing one hour, 0.2 ml. of the mixture is removed and added slowly and with shaking to a mixture of 0.15 ml. 6N hydrochloric acid and 0.1 ml. distilled water. This is allowed to stand for 10 minutes, after which 0.3 ml. of the 20% trichloracetic acid is added drop by drop, with shaking. After standing a further 10 minutes the mixture is centrifuged at high speed for 10 minutes or till the supernatant is clear. From this, 0.37 ml. is removed and placed in a small test-tube graduated at 0.55 ml. The colour is developed as for serum iron by the successive additions of 0.05 ml. of 12N ammonium hydroxide solution, 0.05 ml. saturated sodium acetate solution, approximately 0.01 ml. phenanthroline solution, and 0.3 mg. sodium hydrosulphite, mixing well after each addition, and finally making up to 0.55 ml. The reading is made in the micro cell of the Spekker absorptiometer as before.

Calculation.—The total iron-binding capacity is obtained by reading off the amount of iron on the standard iron graph and multiplying by the factor 1.76.

$$\text{Total iron-binding capacity } (\mu g./100 \text{ ml.}) = R \times \frac{0{\cdot}235}{0{\cdot}20} \times \frac{0{\cdot}75}{0{\cdot}50}$$
$$= R \times 1{\cdot}76$$

where R = amount of iron read off on the graph

Correlation of Iron Content and Colour Development

Fig. 1 shows the linear relation between the Spekker readings and iron content between zero

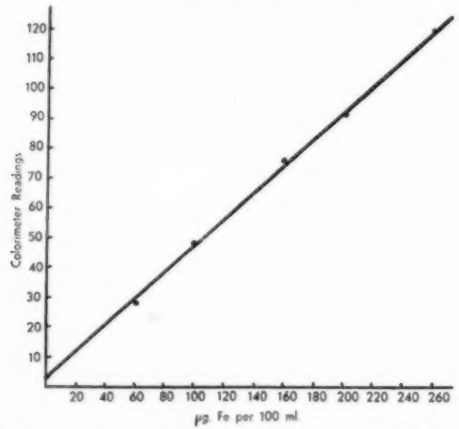


FIG. 1.—Graph of correlation between iron content and colorimeter readings.

and 260 μg. iron/100 ml., a range covering all the readings required for the values of serum iron and iron-binding capacity which have been found.

Accuracy of the Method

In general, there was no difficulty in obtaining good duplicate results. Replicate estimations of serum iron and total iron-binding capacity on 0.2 ml. amounts of the same serum gave the following results.

	Mean (μg./100 ml.)	Range (μg./100 ml.)	Standard Deviation (μg./100 ml.)	Standard Error of Mean (μg./100 ml.)
Serum iron ..	129·6 (5 results)	125–135	±3·6	±1·6
Total serum iron-binding capacity	361 (6 results)	343–369	±9·7	±4·0

In order to obtain reproducible results with these very small amounts of iron it is best to work in an atmosphere as free of dust as possible. In our case the conditions were far from ideal, and to reduce contamination all reagents and glassware were kept, and as far as possible all manipulations carried out, under a large perspex cover. The small test-tubes in which precipitation and development of colour were carried out were always kept capped with tin foil except when reagents were added or aliquots removed. Even with all these precautions occasionally high results were obtained outside the limit of experimental error. These can be ascribed either to random sampling of impurity in the sodium hydrosulphite or to random contamination with dust particles containing traces of iron.

Effect of Free Haemoglobin on Serum Iron and Iron-binding Capacity

The purpose of this investigation was to devise a method applicable to the small amounts of blood obtainable by heel or finger prick in infants and children, and since such specimens of serum would inevitably be somewhat haemolysed it was important to verify the observations of Heilmeyer and Plötner (1937) and Vahlquist (1941) on haemolysed sera. These authors showed that the liberation of iron from haemoglobin by 6N hydrochloric acid was too small to affect the serum-iron estimation. Venndt (1940) showed that this liberation was greatly decreased if the serum containing haemoglobin was first saturated with carbon monoxide. Laurell (1947) confirmed this result and concluded that the presence of free haemoglobin in concentrations up to 380 mg. % in serum affected neither the serum-iron estimation nor the serum iron-binding capacity again provided that the serum was first treated with carbon monoxide, although he pointed out that the values for iron-binding capacity tended to be too high with strongly haemolytic sera. The following experiments were performed to verify these observations.

A solution of haemoglobin was prepared by lysing washed red blood corpuscles with water, and sera containing 70 mg., 140 mg., and 280 mg. per 100 ml. were obtained by adding suitable amounts of this solution to a specimen of serum free from haemoglobin. These sera were all saturated with pure carbon monoxide. Duplicate estimations of serum iron and iron-binding capacity were carried out on the original serum and the three sera containing varying amounts of free haemoglobin. From the results obtained (Table I) it may be concluded that the presence of haemoglobin in amounts up to 140 mg. per 100 ml. of serum

TABLE I

ESTIMATION OF SERUM IRON AND IRON-BINDING CAPACITY IN THE PRESENCE OF HbCO

Amount of Hb in Serum (mg./100 ml.)	Serum Iron (μg./100 ml.)	Total Iron-Binding Capacity (μg./100 ml.)
0	130	361
70	131	375
140	129	379
280	159	408

(equivalent to approximately a 1% haemolysis of the red corpuscles) will not affect the serum iron estimation, providing the haemoglobin is converted to carboxyhaemoglobin. Above this amount the serum iron values tend to be too high. This also applies to the serum iron-binding capacity, within the limits of experimental error. The results therefore confirm those of previous investigators.

Serum Iron and Total Iron-binding Capacity of Serum in Normal Adults

These were estimated in a group of 40 adults, 20 males and 20 females.

Material.—The subjects were between the ages of 18 and 45 years. No attempt was made to take the specimens of blood under fasting conditions or at set times. Samples of capillary blood were taken from ear or finger, and the serum was separated within an hour of collection. Before estimating serum iron the serum was frozen for 24 hours or longer, as recommended by Laurell (1947). Many of the specimens contained some free haemoglobin, judged by naked-eye examination. All such sera were saturated with pure carbon monoxide before analysis. Any samples showing more than 1% haemolysis, as estimated by comparison with serum containing known amounts of haemoglobin, were rejected as being unsuitable for estimation.

Haemoglobin was estimated by the alkaline haematin method, using the Spekker absorptiometer.

Experimental Results

In Table II are shown the results obtained in males and females. They agree well with other published figures, as shown in Table III, which summarizes the results obtained in this and previous investigations of normal adults in which both serum iron and iron-binding capacity have been determined. As might be expected, our results agree best with those of Laurell (1947). There are certain discrepancies between the results obtained by different observers. Most agree that the mean value for serum iron in males is higher than in females, although Cartwright and Wintrobe (1949) found no significant difference. Our results show a higher mean serum iron for males than for

TABLE II

SERUM IRON AND TOTAL IRON-BINDING CAPACITY IN NORMAL ADULTS

	Males				Females			
No.	Serum Iron (μg./100 ml.)	Total Iron-binding Capacity (μg./100 ml.)	% Saturation	Hb (g.%)	Serum Iron (μg./100 ml.)	Total Iron-binding Capacity (μg./100 ml.)	% Saturation	Hb (g.%)
1	109	387	28·2	14·2	110	335	32·8	—
2	76	298	25·5	16·3	82	338	24·3	13·3
3	142	290	49·0	—	95	370	25·7	13·0
4	140	298	47·0	15·7	173	338	51·2	12·1
5	109	334	32·6	15·4	106	266	39·9	13·9
6	133	373	35·7	14·5	93	311	29·9	15·4
7	158	312	50·6	15·8	90	386	23·3	13·2
8	150	306	49·0	14·4	134	275	48·7	14·1
9	140	249	56·2	16·0	126	316	39·9	12·9
10	116	249	46·6	16·4	122	393	31·0	14·4
11	156	302	51·7	15·2	60	429	14·0	12·1
12	132	268	49·3	16·9	128	268	47·8	14·4
13	110	327	33·6	15·5	88	274	32·1	13·9
14	120	266	45·2	15·8	64	204	31·4	14·7
15	108	272	39·7	15·4	134	282	47·5	14·5
16	152	274	55·5	15·4	88	328	26·8	12·7
17	130	304	42·8	14·9	84	355	23·7	12·6
18	132	314	42·1	15·8	132	308	43·9	13·2
19	105	378	27·8	15·8	107	341	31·4	11·1
20	102	286	35·7	16·3	62	282	22·0	13·6
Mean	126·0	304·3	42·2		103·9	320	33·3	
Range	76–158	249–387	25·5–56·2		60–173	204–429	14·0–51·2	
S.D.	±21·5	±39·8	±9·4		±28·9	±52·8	±10·4	
S.E. of mean	±4·8	±8·9	±2·1		±6·5	±11·8	±2·3	

TABLE III

SERUM IRON AND IRON-BINDING CAPACITY IN NORMAL ADULTS

Investigator	No. of Cases	Serum Iron (μg./100 ml.)		Total Iron-binding Capacity (μg./100 ml.)		% Saturation	
		Mean	Range	Mean	Range	Mean	Range
Laurell (1947)	61 M	124	70–214	315	254–406	39·4*	21·0–81·1*
	39 F	108	57–196	315	245–395	34·5	14·4–66·0*
Cartwright and Wintrobe (1949) ..	15 M	127	79–196	347	306–396	36	24–49
	15 F	123	101–164	371	316–429	33	26–42
Rath and Finch (1949)	15 M	106	81–147	311	254–432	34	30–44
	15 F	94	72–130	288	224–415	33	22–44
Ventura and Klopper (1951) ..	25 F	111	88–131	328	241–430	33·7	25–44*
Present investigation	20 M	126	76–158	304	249–387	42·2	25·5–56·2
	20 F	104	60–173	320	204–429	33·3	14·0–51·2

* Calculated from authors' data.

females, which is statistically significant. There is no such clear-cut difference in the case of the total iron-binding capacity. Laurell (1947) could detect none between males and females in this respect, Rath and Finch (1949) found the total iron-binding capacity somewhat higher in males than in females, whereas Cartwright and Wintrobe (1949) found the reverse. Our results agree rather with those of the last, since we also found a higher mean iron-binding capacity in females than in males. In view of these discordant findings it is difficult to estimate the significance of these differences in total iron-binding capacity, especially when the wide spread of results, as well as the relatively small numbers in each group, are considered. In all investigations so far reported the ratio of serum iron to total iron-binding capacity (percentage saturation) is greater for males than for females, but in two series the difference does not appear to be significant. In the present investigation the percentage saturation is significantly greater for males than for females, the difference being 9%, a value larger than that calculated from Laurell's figures, which was only 5%.

Serum Iron and Iron-binding Capacity in Disease

A number of estimations of serum iron and iron-binding capacity have also been performed in hypochromic anaemia and haemochromatosis, as well as in two cases of transfusion haemosiderosis. The results, which are similar to those obtained by other investigators, are shown in Table IV.

TABLE IV

SERUM IRON AND IRON-BINDING CAPACITY IN PATHOLOGICAL CONDITIONS

	Sex	Serum Iron (μg./100 ml.)	Total Iron-Binding Capacity (μg./100 ml.)	% Saturation	Hb (g./100 ml.)
		Hypochromic Anaemia			
1	F	20	370	5·4	6·8
2	F	43	442	9·7	4·9
3	F	21	540	3·9	8·0
4	F	39	372	10·5	5·5
5	F	39	376	10·4	10·2
6	F	46	476	9·7	9·5
7	M	21	382	5·5	7·3
8	M	20	402	5·0	9·3
9	M	45	371	12·2	5·9
10	M	37	375	7·8	5·6
		Haemochromatosis			
1	M	243	239	100	
2	M	240	251	96	
3	M	293	303	97	
4	M	257	256	100	
5	M	255	249	100	
		Transfusion Haemosiderosis			
1	F	381	378	100	
2	F	305	322	95	

Hypochromic Anaemia.—The results in 10 cases show that the range of serum iron is lower than the normal, while that of the iron-binding capacity falls in the upper limit of the normal range. The percentage saturation is always low, in this series below 12.2%, compared with a mean percentage saturation of 33.3% for females and 42.2% for males.

Haemochromatosis.—The results show that in this condition the serum iron is always greater than normal, whilst the iron-binding capacity is within the lower limits of normal. What is of greater significance, however, is that the percentage saturation appears to be always 100% or nearly so. This may also occur in other conditions, pernicious anaemia, aplastic anaemia (primary refractory anaemia), Mediterranean anaemia, and transfusion haemosiderosis. Haemochromatosis is perhaps not so uncommon as might be supposed, and the results indicate that these estimations afford a relatively easy laboratory test in suspected cases of this disease.

Both cases of transfusion haemosiderosis due to multiple transfusions for aplastic anaemia showed 100% or nearly 100% saturation of the iron binding capacity.

Summary

A micro-method for the estimation of serum iron and iron-binding capacity, requiring only 0.2 ml. serum for each, is described.

Using this method, the serum iron and iron-binding capacity in a group of normal adults, 20 males and 20 females, as well as in a group of cases of haemochromatosis and hypochromic anaemia, have been determined.

We wish to thank Dr. M. Lubran and Dr. N. F. Coghill for specimens of sera from patients with haemochromatosis and Drs. Hopkins and Bernstock for those from patients with hypochromic anaemia.

The above forms part of an investigation undertaken by Helen M. Mackay and B. Levin, with the assistance of G. Davies and V. G. Oberholzer, who gratefully acknowledge grants from the Medical Research Council.

REFERENCES

Budtz-Olsen, O. E. (1951). *Journal of Clinical Pathology*, **4**, 92.
Burch, H. B., Lowry, O. H., Bessey, O. A., and Berson, B. Z. (1948). *J. biol. Chem.*, **174**, 791.
Cartwright, G. E., and Wintrobe, M. M. (1949). *J. clin. Invest.*, **28**, 86.
Heilmeyer, L., and Plötner, K. (1937). *Das Serumeisen und die Eisenmangelkrankheit*. Jena.
Holmberg, C. G., and Laurell, C.-B. (1945). *Acta physiol. scand.*, **10**, 307.
Laurell, C.-B. (1947). Ibid., **14**, Suppl. 46.
Rath, C. E., and Finch, C. A. (1949). *J. clin. Invest.*, **28**, 79.
Schade, A. L., and Caroline, L. (1946). *Science*, **104**, 340.
Vahlquist, B. C. (1941). *Acta paediat., Uppsala*, **28**, Suppl. 5.
Venndt, H. (1940). *Hoppe-Seyl. Z. physiol. Chem.*, **283**, 162.
Ventura, S., and Klopper, A. (1951). *J. Obstet. Gynaec. Brit. Emp.*, **58**, 173.

J. clin. Path. (1952), **5**, 317.

TRUE URIC ACID VALUES

BY

J. MAXWELL JOHNSTONE

From the S.A. Courtauld Institute of Biochemistry, the Middlesex Hospital Medical School, London

(RECEIVED FOR PUBLICATION APRIL 15, 1952)

The estimation of uric acid in blood is often unsatisfactory, and substances commonly present in blood, other than uric acid, may react with the reagents to give false values. This difficulty may be overcome by the use of uricase (Blauch and Koch, 1939; Bulger and Johns, 1941; Block and Geib, 1947), although in these methods some uric acid is probably lost during protein precipitation. The error due to unrelated substances may be equivalent to 0.32–1.54 mg. uric acid/100 ml. (Block and Geib, 1947; Blauch and Koch, 1939).

This paper describes uric acid values found in normal and pathological bloods before and after treatment with uricase, and indicates the error due to non-uric acid reacting substances.

Method

The procedure of Block and Geib (1947) has been modified by substituting for their arsenophosphotungstic acid reagent, which gives too weak colours, the colour-developing reagents described by Brown (1945). By this method a linear relationship is obtained between uric acid concentration and optical density up to about 12 mg. uric acid/100 ml. plasma.

As the intensity of the colour gradually increases even after one hour, all colours were read precisely the same number of minutes after adding the reagents. This was achieved by adding the phosphotungstic acid reagent to successive tubes at one-minute intervals.

Reagents.—The following were used: (1) uricase powder (Buchanan, Block, and Christman, 1945); (2) uricase extract (Block and Geib, 1947); (3) 10% (w/v) sodium tungstate; (4) 2/3N H_2SO_4; (5) lithium chloride solution, made by dissolving 7.5 g. lithium chloride in water to which 35 ml. concentrated HCl has been added, and diluting to one litre; (6) 2.9% (w/v) silver nitrate; (7) 50% (w/v) urea; (8) 12% (w/v) recrystallized sodium cyanide (made immediately before use, otherwise blanks will have too great a density); (9) phosphotungstic acid reagent (Folin, 1934); (10) stock uric acid solution (Folin, 1930), but without methyl orange; (11) standard uric acid solutions made by diluting 2, 4, 6, and 8 ml. each of stock uric acid solution to 1,000 ml., which, when used as described below, are equivalent to 2, 4, 6, and 8 mg. uric acid/100 ml.; (12) N/3 HCl.

Procedure

Non-uric Acid Residual Colour.—To 2 ml. of plasma 2 ml. of uricase extract and 12 ml. of water are added. The solution is incubated for two hours in a water-bath at 45° C. and then the proteins are precipitated, adding 2 ml. each of 10% sodium tungstate and 2/3N H_2SO_4. After 20 to 30 minutes the solution is centrifuged and the supernatant filtered through a No. 54 Whatman filter paper. To 8.0 ml. of filtrate 0.8 ml. of lithium chloride solution and 0.8 ml. of silver nitrate solution are added, mixed, and centrifuged at once. In this order, 2 ml. of 50% urea, 2 ml. of sodium cyanide solution, and 1 ml. of phosphotungstic acid reagent are added to 6.0 ml. of the supernatant; the solution is mixed, and then diluted to 15 ml. with water.

Uricase Blank.—Uricase extract, 2 ml., and 14 ml. of water are incubated for two hours at 45° C. and carried through the above procedure.

Total Colour.—Water, 7 ml., and 1 ml. each of 10% sodium tungstate and 2/3N H_2SO_4 are added to a tube containing 1 ml. of plasma. After 20 to 30 minutes the solution is centrifuged and the supernatant filtered as above. To 4.0 ml. of filtrate 0.8 ml. each of lithium chloride solution and silver nitrate solution are added, and the resulting solution mixed and centrifuged immediately. Then 2 ml. of 50% urea, 2 ml. of 12% sodium cyanide solution, and 1 ml. of phosphotungstic acid reagent are added to 3.5 ml. of the supernatant and made up to 15 ml. with water.

Standards.—To 2.5 ml. of each of the standard solutions 1 ml. of N/3 HCl, 2 ml. of 50% urea, 2 ml. of 12% sodium cyanide, and 1 ml. of phosphotungstic acid reagent are added, and diluted to 15 ml. with water. Throughout this procedure a reagent blank is run.

The colour intensities are read after 45 to 50 minutes in a Spekker absorptiometer, using Ilford red No. 608 filters.

Calculation.—The readings, corrected for blanks, are converted to mg. uric acid by the calibration curve. The non-uric acid values must be halved, as 2 ml. of plasma were originally used for these estimations.

Results

Uric acid values in 105 plasma samples are shown in Table I.

TABLE I
MEAN TOTAL URIC ACID VALUES AND RANGES IN EACH TYPE OF CASE INVESTIGATED

Condition		No. of Cases	Mean Total Uric Acid Value (mg.%)	Range of Total Uric Acid Values (mg.%)
Normal	Men	17	4·61 ± 0·24	2·80– 7·00
	Women ..	21	3·75 ± 0·22	2·00– 6·50
Uraemia		14	7·49	5·30–11·30
Gout		8	7·80	4·25–10·10
Rheumatoid arthritis ..		7	3·88	3·05– 4·80
Diabetes mellitus		10	3·84	2·60– 6·05
Leukaemia		8	6.92	2·65–26·00
Carcinoma		4	4·27	3·00 –6·90
Miscellaneous*		16	4·12	2·25– 9·00

* This group comprised two cases each of hypothyroidism and obstructive jaundice, and one case each of intestinal obstruction, cirrhosis, prostatic enlargement, albuminuria, lupus vulgaris, Addison's disease, low renal threshold, hepatosplenomegaly, ulcerative colitis, chronic urinary infection, hypopituitarism, and gangrene of the foot.

Normals.—In 38 normal people the mean total uric acid value, irrespective of sex, was 4.14 ± 0.16 mg./100 ml. and the mean non-uric acid reacting substances value 0.072 ± 0.005 mg./100 ml. There was a significant difference (Table I) between the mean total uric acid values for men and women in this group ($t = 2.836$, $n = 38$, $P < 0.01$).

Uraemia.—Of 14 uraemic patients the total uric acid value in eight was over 7 mg./100 ml., while in 11 the value of the non-uric acid reacting substances was over 0.20 mg./100 ml., the highest reading, from a case of polycystic renal disease, representing 2.00 mg. uric acid/100 ml.

Leukaemia.—Of eight cases, acute and chronic, seven showed normal values. The eighth, a child in the terminal stages of acute lymphatic leukaemia, had a total uric acid value of 26 mg./100 ml. plasma and the non-uric acid reacting substances value represented 8.2 mg. uric acid/100 ml. plasma.

Rheumatoid Arthritis, Diabetes Mellitus, and Carcinoma.—All values were within the normal ranges in seven cases of rheumatoid arthritis, 10 cases of diabetes mellitus, and four cases of carcinoma.

Gout.—In six of the eight cases the total uric acid value was over 7.00 mg./100 ml. All non-uric acid reacting substances values were normal.

Miscellaneous.—Only one, a patient with obstructive jaundice, had a raised total uric acid value. One patient with obstructive jaundice, one with hepatic cirrhosis, and one with albuminuria of obscure origin had non-uric acid reacting substances with values greater than 0.20 mg./100 ml.

Discussion

The nature of the non-uric acid reacting substances is obscure. In this work it was found that dihydric phenols, such as resorcinol, metol, guaiacol, and pyrogallol, gave strong colours with the reagents used: 1 mg./100 ml. solutions of resorcinol and guaiacol, when treated as plasma samples, gave colours representing 3.6 and 3.08 mg. uric acid/100 ml. respectively. However, the phenolic extract of a sample of normal urine (10 minutes' hydrolysis with dilute acid and 12 hours' benzene extraction) accounted for only 0.08 mg. of the non-uric acid reacting substances value of 7.00 mg./100 ml. urine.

There is no correlation between the values for total uric acid and non-uric acid reacting substances. In the cases of gout all the raised uric acid values were accompanied by normal non-uric acid reacting substances values. On the other hand, in many of the uraemic patients there were raised values for both the non-uric acid reacting substances and true uric acid.

In routine laboratory practice determination of total plasma uric acid values by Brown's method (1945) is quite satisfactory. In the majority of cases it will give values not more than 0.20 mg./100 ml. greater than the true uric acid value. It is principally in cases of considerable renal damage that the error may be greater.

Summary

A slightly modified method for the determination of true uric acid values using uricase is described.

The mean total plasma uric acid value in 38 normal people was 4.14 ± 0.16 mg./100 ml.

The total uric acid value, estimated by the method of Brown (1945), is normally less than 0.20 mg./100 ml. greater than the true uric acid value.

I should like to express my gratitude to Professor E. C. Dodds for permission to do this work and to those who provided me with some of the blood samples for investigation.

REFERENCES

Blauch, M. B., and Koch, F. C. (1939). *J. biol. Chem.*, **130**, 443.
Block, W. D., and Geib, N. C. (1947). Ibid., **168**, 747.
Brown, H. (1945). Ibid., **158**, 601.
Buchanan, O. H., Block, W. D., and Christman, A. A. (1945). Ibid., **157**, 181.
Bulger, H. A., and Johns, H. E. (1941). Ibid., **140**, 427.
Folin, O. (1930). Ibid., **86**, 179.
—— (1934). Ibid., **106**, 311.

J. clin. Path. (1952), **5**, 319.

THE CARBOHYDRATES IN NORMAL AND ABNORMAL CEREBROSPINAL FLUID

BY

MARJORIE D. EASTHAM AND KENNETH R. KEAY

From the Department of Biochemistry, University College, Dundee, and the Department of Paediatrics, the Medical School, University of St. Andrews

(RECEIVED FOR PUBLICATION APRIL 8, 1952)

Determination of the sugar content forms an essential part in the complete examination of cerebrospinal fluid. The sugar determined is generally considered to be mainly glucose (Harrison, 1947).

Hubbard and Russell (1937), using a resorcinol colorimetric estimation, found a ketose in varying amounts which they thought was probably fructose.

In the present study a discrepancy was observed between the value of reducing compounds determined in cerebrospinal fluid by the method of Hagedorn and Jensen and that of Somogyi (1945) comparable to that observed by Somogyi and Kramer (1928) in blood sugar determination. It was believed that paper partition chromatography might throw some light on the reasons for this discrepancy. A comparative study has been made in 100 cerebrospinal fluids using four different methods of examination for reducing sugars: (1) Benedict's test; (2) the Hagedorn–Jensen estimation; (3) Somogyi copper-phosphate estimation; (4) paper partition chromatography, qualitative (Partridge, 1948) and quantitative (Flood, Hirst, and Jones, 1947).

It is generally agreed that the various forms of meningitis are accompanied by a fall in the amount of reducing substance in cerebrospinal fluid below 45 mg./100 ml. (MacCarthy and Mann, 1950). In certain cases of meningitis, however, values greater than this have been reported. With the advent of streptomycin treatment for tuberculous meningitis early diagnosis is of first importance, and it seemed useful to know (1) if the discrepancy between methods was extensive amongst normals and existed also in the anomalous meningitis cases, and (2) what was the nature of the reducing substance.

Methods

Preservation of Specimens.—In accordance with the work of Cohen (1936), whose experimental results on preservation were first confirmed on 17 samples, specimens were collected under sterile conditions and retained at 0° C. Occasional specimens contained sodium fluoride, which was found to be neither detrimental nor necessary. Specimens were obtained mainly by lumbar puncture; a few from cisternal and lumbar punctures were compared.

Hagedorn–Jensen and Benedict Methods.—These were carried out according to Harrison (1947).

Somogyi Method.—This method (Somogyi, 1945) was carried out using the iodate–iodide titration method.

Qualitative Chromatography.—In this the method finally used was as follows: 2 ml. of sample was evaporated in a 1-in. diameter glass dish to a paste on the water bath at 45° C. and then completely dried in a vacuum desiccator. The desiccate was well mixed with 0.2 ml. of methanol and the resulting suspension removed into a 2 × 0.3 cm. agglutination tube. The salt and protein precipitate was allowed to settle into the pointed end overnight at 0° C., this period ensuring the solubility of less soluble sugar amines.

A measured volume of 0.05 ml. of the clear supernatant methanol extract of the sugars was placed on spots in triplicate on No. 3 Whatman paper, paired alongside mixtures of standard sugars.

After irrigation with butanol-acetic acid for 48 to 60 hours the paper was cut vertically into three and the portions sprayed with the three colour reagents: (1) benzidine (Horrocks, 1949); (2) naphthoresorcinol and trichloracetic acid at 90°–100° C. in steam (Partridge, 1948; Forsyth, 1948); and (3) dimethylaminobenzaldehyde and acetylacetone (Morgan and Elson, 1934) in order to differentiate between aldehyde and ketone sugars, sugar amines, and uronic acids.

Grouping of Cases.—The cases were grouped as (I) "normal"; (II) tuberculous and non-tuberculous meningitis; and (III) miscellaneous cases of disease of the central nervous system. Group III will not be discussed in this paper.

I: "Normals."—This group consisted of 50 cases on which the chromatographic, Hagedorn–Jensen, and Benedict methods were all carried out; on 26 of these 50 cases the Somogyi method was also used.

This group included all cases of specimens collected before spinal anaesthesia, together with those of non-cerebrospinal disease in live persons which were normal as regards cell count, protein, Lange gold curve, and "sugar."

It included also a further 17 cases used for fructose estimation by Roe's method (1934) and 10 cases used for glucosamine estimation by the method of Morgan and Elson (1934).

In both the Roe, and Morgan and Elson, colorimetric methods an E.E.L. photoelectric colorimeter with a green filter was used for the measurements. Reagent blanks and a suitable range of standards were made afresh with each set of readings. Analar standard HCl and recently opened or redistilled alcohol were used. Test-tubes of 15×150 mm. were used uncovered during heating.

For quantitative chromatography, as described by Flood *et al.*, bulked samples of 30 ml. were collected and evaporated and the methanol extract used. Each individual sugar was extracted from its horizontal paper strip with methanol and evaporated for identification.

Quantitative estimations showed little of interest except that there was a zone at Rf 0.28–0.30 containing material which reduced both Hagedorn–Jensen and Somogyi reagents but gave no brown spot with benzidine, showing only a white cone. It was not identified.

Crystals from each strip were examined microscopically. From strip Rf 0.14–0.16 crystalline plates were obtained from concentrated HCl with a melting point at 142–6° C. Both phenylhydrazone and osazone were prepared on the microscope slide and compared with those from B.D.H. dextrose. The fructose strip at Rf 0.23 yielded a similar osazone and a methylphenylhydrazine compound of melting point 158° C.

A further bulked sample of approximately 100 ml. was evaporated as collected and retained at 0° C. for preparation of glucosamine nitrobenzylidene compound by the method of Jolles and Morgan (1940). It yielded crystals like Christmas trees, with a strong hyacinth odour at a melting point of 180–182° C., 6 mg. in weight, decomposing above this temperature to a brown mass and a white sublimate, similar to those from glucosamine hydrochloride.

The use of bulked samples is open to criticism but individual specimens were chomatographed to ensure normality.

II: Abnormals.—This group includes all the cases where meningeal infection was diagnosed. It has been subdivided into a main group of tuberculous meningitis (IIa) and non-tuberculous meningitis (IIb). Cases of miliary tuberculosis have been included with IIa for purposes of comparison and contrast.

As with the normals in Group I, it was possible to test only a proportion of cases by the Somogyi method owing to insufficiency of the sample, but all were examined by chromatography and the Hagedorn–Jensen method and a number by the fructose estimation of Roe.

Results

Group I.—Fig. 1 shows the subgroup of 26 normals. The Benedict reactions were all red or orange. Somogyi's estimations vary between 35 and 90 mg. with an average of 67 mg./100 ml.

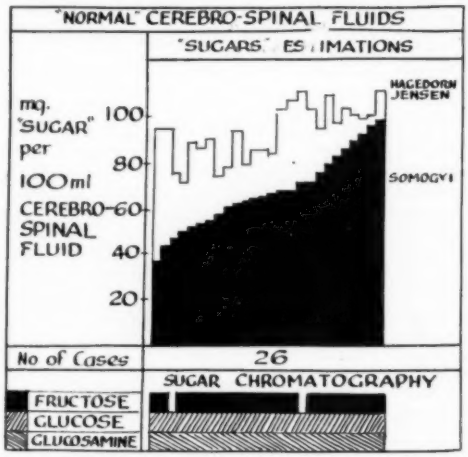


FIG. 1.—Comparison of normal cerebrospinal fluids by three methods of examination for reducing "sugars" presented in ascending order of Somogyi estimation. Sugars in chromatogram diagrams are represented as being normal, absent, or diminished considerably.

Hagedorn–Jensen figures vary between 66 and 112 mg. with an average of 88 mg./100 ml.

In the chromatograms three spots, glucosamine hydrochloride and base (Rf 0.14–0.16), glucose (Rf 0.18), and fructose (Rf 0.23), are present in all but two of the samples, in which fructose was missing. (The standard glucosamine hydrochloride used for purposes of comparison also always produced two spots, hydrochloride and base in butanol acetic acid.)

No absolute relationship exists between the Somogyi and Hagedorn–Jensen figures. When the Somogyi estimations are arranged as in Fig. 1 in ascending order, the Hagedorn–Jensen figures are *not* in ascending order: the Hagedorn–Jensen method estimates other unsaturated compounds, for example, those containing double bonds, as well as sugars, while Somogyi claims that his method estimates most nearly the true sugar content. The figures in this group thus show an average of 25% non-sugars.

In a further 24 normal samples in which Hagedorn–Jensen determinations only were carried out the chromatographic results were similar.

In no case out of the total 50 was glucose absent and fructose present. In the six cases where no fructose appeared the disease diagnosis was diverse (including two cases of bronchopneumonia, one each of epilepsy, petit mal, tonsillitis, and convulsions).

When fructose was *not* apparent on the chromatogram, the fructose content by Roe's method was

less than 1 mg./100 ml. of cerebrospinal fluid. A very few specimens showed definite brown spots on the chromatogram at Rf 0.28 (the Rf of xylose) and four specimens gave evidence of a keto form of pentose at the Rf of the urine sugar keto-xylose, 0.29. In no specimen was a spot for reduced ascorbic acid noticed.

Group II.—Fig. 2 represents tuberculous meningitis cases in hospital. The 19 specimens from the 14 cases under treatment were taken in the resting periods between streptomycin treatment.

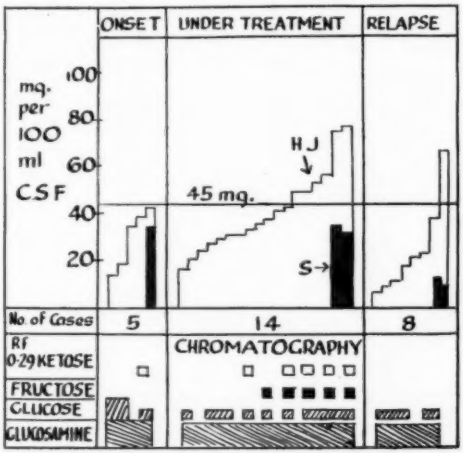


FIG. 2.—Comparison of tuberculous meningitis cerebrospinal fluids by three methods of examination for reducing sugars as in Fig. 1.

The Hagedorn–Jensen figures are arranged in ascending order. In this group they are mainly below 45 mg. while the few anomalous cases above 45 mg. gave Somogyi figures below 45.

In the corresponding chromatograms much of the normal sugar is absent. From only five specimens traces of the fructose spot appeared; from all the others it was absent. Glucose spots were smaller and in certain early cases had disappeared completely.

In addition to the 27 tuberculous meningitis specimens charted in Fig. 2 a further 14 specimens not shown on Fig. 2 were examined: (1) seven "tests of cure," all cases with a cell count under 10, and (2) seven cases of miliary tuberculosis with no meningeal involvement. In all cases the Hagedorn–Jensen figure was over 45 mg. and in all chromatograms all three normal spots were present. In only one case did the ketose (Rf 0.29) occur.

Fig. 3 represents the few non-tuberculous meningitis cases. Column 1 shows two cases of aseptic meningitis examined at the onset. For Case 1, first and sixth day specimens were examined and for Case 2, those of the first, second, third, fifth, and thirteenth days. (Penicillin was administered on the second.) Case 3 is of pneumococcal, Cases 4 and 5 of meningococcal, and Case 6 of benign lymphocytic meningitis.

Somogyi estimations in Cases 1 and 2 are well below 45 mg. until the thirteenth day (Case 2), whilst the Hagedorn–Jensen results are consider-

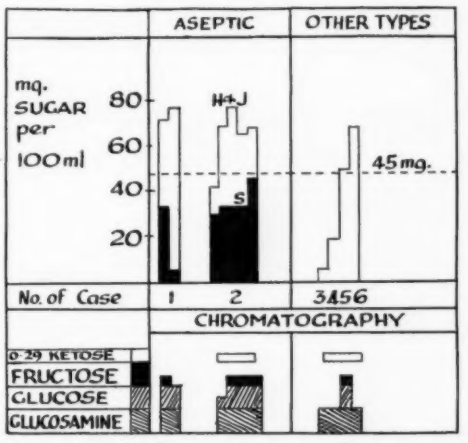


FIG. 3.—Comparison of cerebrospinal fluids of various types of non-tuberculous meningitis for reducing sugars by three methods as in Fig. 1.

ably above 45 mg. except on the first day. The disappearance of fructose and decrease of glucose from the chromatogram is similar to that in tuberculous meningitis; there is also an occasional trace of a second ketose.

Fructose Estimations (Roe Method).—Groups I and II are collected on one diagram (Fig. 4) for purposes of comparison.

Simultaneous chromatography and estimation of ketose by Roe's resorcinol method showed that the ketose estimated in the normal specimens was fructose. In the pathological specimens the method would estimate as fructose the trace of a second ketose which was present in three of the specimens in column 3. Column 2 indicates that the normal range for fructose was 1.5 to 7.0 mg. The one exception was the lowest, a hypoglycaemia in which the blood sugars had fallen to 28 mg. In tuberculous meningitis (column 3) all fructose

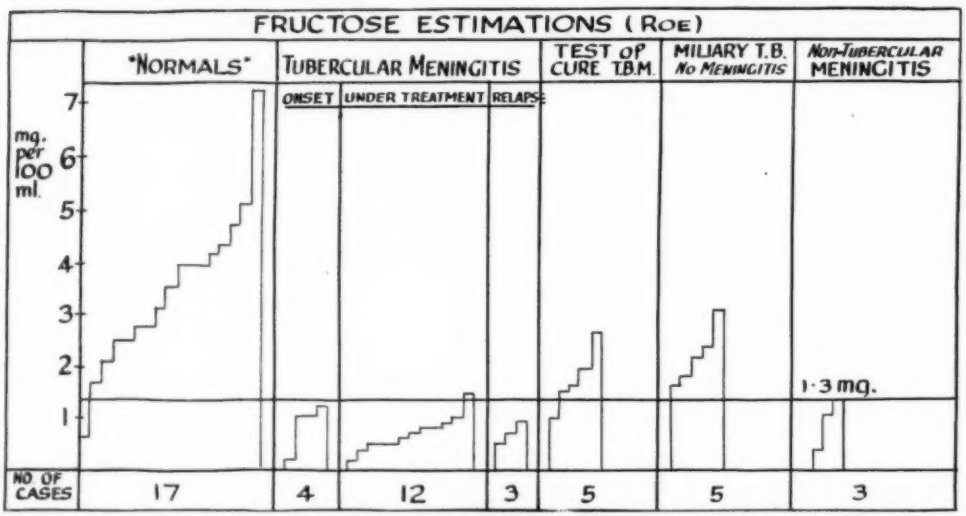


FIG. 4.—Comparison of fructose content of normal and tuberculous meningitis cerebrospinal fluid by Roe's method.

figures were below 1.5 mg. and in "onset" and "relapse" ranged from 0.2 to 1.0 mg./100 ml.

In columns 6 and 7 the "tests of cure" of tuberculous meningitis and miliary tuberculosis cases, the fructose range is similar to the normal range and no second ketose was found. It seems fair to conclude that the ketose estimated by Hubbard and Russell (1951) was fructose in the normal material and that the occurrence of the second ketose is pathological and occasional.

TABLE I
HEXOSAMINE* ESTIMATION (MORGAN-ELSON METHOD)

	Hexosamine (mg. per 100 ml.)
Range of normals (10 cases)	5–18 (mean 9)
Range of tuberculous meningitis:	
Under treatment (10 cases)	0–12 (mean 7)
Yielding to treatment (10 cases) ..	3–20 (mean 9)
Relapse (3 cases)	6–16 (mean 10)

* Calculated as glucosamine.

In view of the criticism of Horowitz, Ikawa, and Fling (1950) of the effect of ketose and lysine in large quantities on the Morgan-Elson estimation the actual figures are not given too much weight except to emphasize that hexosamine estimation is of little value in the diagnosis or prognosis of meningitic disease.

In contrast to fructose, the hexosamine values obtained showed no great divergence between normal specimens and those from meningeal disease.

Effect of Antibiotics on the Chromatographic Method.—Owing to increased permeability of the blood brain barrier, levels of penicillin and streptomycin detectable in the cerebrospinal fluid have been found in meningitis (Rosenberg and Sylvester, 1944). The possible effect of streptomycin on the chromatogram was therefore investigated.

It was found that streptomycin itself did not move forward in butanol acetic, whilst the acid-hydrolysed products of streptomycin gave a brown sugar spot with benzidine at Rf 0.10 and a reaction with the Morgan-Elson reagent at approximately the same Rf, probably the known acid hydrolysis product streptobiosamine. No spots within the range of a normal or tuberculous meningitis cerebrospinal fluid chromatogram were observed.

Streptomycin gave no positive reactions with the Roe colorimetric estimation for fructose, so this method might have advantages over other sugar estimation methods as an aid to prognosis in meningitis.

Discussion

Source of Glucosamine and Fructose.—Two sources of the hexosamine were thought possible: (1) that it might be combined in a polysaccharide or mucoprotein as in blood serum (Shetlar, Foster, Kelly, and Everett, 1948); (2) that transamination from amino-acids on to the sugars was taking place.

To investigate the first supposition the precipitate from methanol extracts from the normal speci-

mens was hydrolysed with 15% HCl in sealed tubes for from three to six hours at 100° C. Morgan-Elson estimations and chromatograms of such hydrolysates showed a hexosamine-reacting material at about Rf 0.08 to 0.10, but amounts were always less than 2–3 mg./100 ml. cerebrospinal fluid.

It seems as though there is very little hexosamine containing polysaccharide normally in cerebrospinal fluid. Very enlarged glucosamine spots were found only in the cerebrospinal fluid of pneumonia cases and this may be connected with the already noted high glucosamine polysaccharide values of pneumonia blood sera (Nilsson, 1937). This observation is some slight evidence towards the hypothesis that the blood serum polysaccharide may be providing the hexosamine of the cerebrospinal fluid in an easily freed or free form.

The possibility of the second supposition being part of the truth is indicated by recent work on the chromatography of amino-acid and glucose or fructose mixtures. Obviously more work is required to determine the answer.

Arguing from the swift disappearance of fructose in meningeal infection, it would appear that an enzyme in the cells of the meninges might be responsible for the normal presence of fructose in cerebrospinal fluids. Although the brain selectively uses glucose rather than fructose (Maddock, Hawkins, and Holmes, 1939; Klein, 1944), the latter cannot reasonably all be supplied by the blood, as the fructose content of serum is so very slight and its rate of transfer from blood to brain five to 10 times less than glucose (Klein, Hurwitz, and Olsen, 1946). Furthermore, at least *in vitro*, *Meningococcus* and *Myco. tuberculosis* are not actively glycolytic (Hendry, 1939). It may be that glucosamine is the "easily changeable form of glucose" postulated by Hubbard and Russell as existing in cerebrospinal fluid. Again more work is required, particularly on a comparison between vascular, cisternal, and lumbar punctures. As a corollary to this study it has only been possible to compare a few cisternal and lumbar punctures by chromatography, when little or no difference was observed except in acute and old cases of hydrocephalus or meningitic blocks. No material has been chromatographed from the perivascular spaces of the brain, which might conceivably have a fructose content more nearly that of blood serum.

Estimation Methods.—The results obtained by the Hagedorn–Jensen and Somogyi methods of estimation of reducing substances support the work of Cacioppo and Bevilotti (1945), except that the ratio of the means is higher in this work. In meningitis it would appear that there is less likelihood of encountering the anomalous cases showing over 45 mg. when the Somogyi method is used.

Summary

The copper reduction methods of Somogyi give lower results than the Hagedorn–Jensen method in cerebrospinal fluid sugar estimations in both normal and meningitic cases.

Paper chromatography has demonstrated that there are at least three reducing substances present in cerebrospinal fluid from normal people and from cases of non-cerebrospinal disease. These substances are glucose, fructose, and glucosamine. In addition there are substances not yet identified, with a reducing action on both Hagedorn–Jensen and Somogyi reagents.

Fructose values fall at the onset of tuberculous meningitis, remain low during illness, regain low normality on recovery, and fall rapidly in relapse. Their estimation would appear to be of value in helping the rapid diagnosis of meningeal disease.

The range of hexosamine values does not appear to differ significantly in meningeal disease from that of normal values, at least by the Morgan-Elson method of estimation.

We wish to thank Dr. R. P. Cook, of the Biochemical Department, University College, Dundee, where this work was carried out, and also the Bacteriology Department for specimens and information.

All tuberculous meningitis specimens in Fig. 4 and 27 of the 46 specimens in Group II were investigated with the co-operation and diagnostic assistance of Dr. W. M. Jamieson, Physician Superintendent, King's Cross Isolation Hospital, Dundee, and Dr. J. H. Prain, of the Pathology Department, Royal Infirmary, Dundee.

References

Cacioppo, F. C., and Bevilotti, V. B. (1945). *Boll. Soc. ital. Biol. sper.*, **20**, 244.
Cohen, H. (1936). *Quart. J. Med.*, **5**, 159.
Flood, A. E., Hirst, E. L., and Jones, J. K. (1947). *Nature, Lond.*, **160**, 86.
Forsyth, W. G. C. (1948). Ibid., **161**, 239.
Harrison, G. A. (1947). *Chemical Methods in Clinical Medicine*, 3rd ed. London.
Hendry, E. (1939). *Arch. Dis. Child.*, **14**, 307.
Horowitz, H. N., Ikawa, M., and Fling, M. (1950). *Arch. Biochem.*, **25**, 226.
Horrocks, R. H. (1949). *Nature, Lond.*, **164**, 444.
Hubbard, R. S., and Russell, N. M. (1937). *J. biol. Chem., Lond.*, **119**, 647.
Jolles, Z. E., and Morgan, W. T. J. (1940). *Biochem. J.*, **34**, 1183.
Klein, J. R. (1944). *J. biol. Chem.*, **153**, 295.
—— Hurwitz, R., and Olsen, N. S. (1946). Ibid., **164**, 509.
Maddock, S., Hawkins, J. E., and Holmes, E. (1939). *Amer. J. Physiol.*, **125**, 551.
MacCarthy, D., and Mann, T. P. (1950). *Lancet*, **1**, 341.
Morgan, W. T. J., and Elson, L. A. (1934). *Biochem. J.*, **28**, 988.
Nilsson, I. (1937). *Biochem. Z.*, **291**, 254.
Partridge, S. M. (1948). *Biochem. J.*, **42**, 238.
Roe, J. H. (1934). *J. biol. Chem.*, **107**, 15.
Rosenberg, D. H., and Sylvester, J. C. (1944). *Science*, **100**, 132.
Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R. (1948). *Proc. Soc. exp. Biol., N.Y.*, **67**, 125.
Somogyi, M., and Kramer, H. V. (1928). *J. biol. Chem.*, **80**, 733.
—— (1945). Ibid., **160**, 61.

J. clin. Path. (1952), **5**, 324.

OX-CELL HAEMOLYSINS IN HUMAN SERUM

BY

G. B. LEYTON

From the Provincial Laboratory, Winnipeg, Manitoba, Canada

(RECEIVED FOR PUBLICATION MAY 24, 1952)

Ever since Ehrlich and Morgenroth (1899, 1900) showed that the naturally occurring lysins were specific antibodies many workers have shown sporadic interest in the power of human sera to agglutinate and haemolyse the erythrocytes of many other vertebrae. Forssman (1911) showed that it was possible to produce haemolysins for sheep corpuscles in the sera of rabbits by injecting them with organs obtained from guinea-pigs, and that the titres of the sera were much higher than were ever found with naturally occurring antibodies. This was the first description of what are now known as heterophile antibodies, and Forssman's work has been confirmed and amplified by many workers, including Taniguchi (1920, 1921, 1922), Davidsohn (1929, 1930), Davidsohn and Ramsdell (1929), and Browning (1931). All these workers concerned themselves with the theoretical aspect of the antibody and the differentiation of one type of heterophile antibody from another. Most of these workers used agglutination rather than haemolysis as a criterion for the presence of the antibodies, and sheep cells were by far the commonest erythrocyte used in their experiments. Paul and Bunnell (1932) observed that the serum of patients suffering from glandular fever (infectious mononucleosis) showed the presence of agglutinating antibodies for sheep cells to a high titre. Ever since the discovery of this phenomenon the power of patients' sera to agglutinate sheep cells has been used to help in the diagnosis of glandular fever. Changes in the technique of the original test and the introduction of guinea-pig kidney and boiled ox cells for differential absorption of the Forssman and pseudo-Forssman antibodies by Stuart, Welch, Cunningham, and Burgess (1936) and Davidsohn (1937) have led to a considerable increase in the accuracy of the results obtained. Although Paul and Bunnell used the agglutinating antibodies as the basis of the test that bears their name, it was immediately realized that haemolysing antibodies were also present against the sheep corpuscles as well as against the corpuscles of some other animals. Bailey and Raffel (1935) conducted experiments to elucidate something of the nature of both the agglutinating and haemolysing antibodies present in three cases of glandular fever. They showed that the haemolysins were active against both sheep and ox cells, but found little agglutination of ox cells with the sera they used. Since then little attention has been paid to haemolysing antibodies found in human sera in general, and practically no mention of their affinity for ox cells in particular. In view of the comparatively scant literature on this well-recognized phenomenon and the varying results obtained, it seemed worth while to investigate the position more fully.

Preliminary experiments showed that classical cases of glandular fever have in their sera a high titre of haemolysing antibodies for ox cells. Using a 2% suspension of red cells, all efforts to show the presence of agglutinating antibodies for ox cells in cases of glandular fever were unsuccessful, although attempts were made with cells suspended in saline, in 30% albumin, in dextran, and in sorbitol and glycerine. A weak agglutination could sometimes be obtained at a titre of 5 and 10 if the tubes were centrifuged after the cells and sera had been incubated for 15 minutes at 37° C. The ox cells were agglutinated after exposure to sera from cases of glandular fever by rabbit anti-human globulin prepared as for the Coombs test, but also ox-cell absorbed. Even this method was not uniformly successful. However, before any comparison of the activity of various sera can be satisfactorily achieved, even by a haemolytic reaction, it is essential to be able to maintain the day-to-day sensitivity of the test within small limits. With this object in view it was decided to investigate something of the nature of the reactions involved.

Technique

In any test employing erythrocytes, complement, and a haemolysin there are clearly three variables. Therefore, if we wish to estimate one of these with any degree of accuracy it is essential that the other

two variables be kept constant or at any rate be so arranged that they do not affect the result of the test. In this investigation it is the haemolysin which is being studied; therefore it was decided first to see how variation in the strength of the red cell suspension and the amount of complement present altered the haemolytic titre of a single specimen of serum. Experiments showed that the haemolytic titre of a human serum for ox cells was inversely proportionate to the concentration of erythrocytes provided that the ratio of erythrocytes to complement was kept constant. When from 1 to 8 M.H.D. (minimum haemolytic doses) of complement were used even slight variation in the amount present altered the haemolytic titre of the serum considerably, but when more than 8 M.H.D. of complement were present no significant alteration of the titre of a serum was observed regardless of how much more complement was present. It was thought that varying the size of the tubes might affect the titres observed, but it was found that a change of at least 50% in the bore of the tubes made no difference to the results.

These experiments showed that it would be possible to maintain the sensitivity of the test at a constant level, but that it was essential to control the strength of the erythrocyte suspension and concentration of complement. It was therefore decided that in all further experiments, unless stated expressly to the contrary, the following technique would be used:

Erythrocytes.—An approximately 2% suspension of red cells was made up and standardized as follows: Ox cells, preserved in glucose citrate and not more than three weeks old, were washed three times with 0.9% sodium chloride, and a suspension a little stronger than 2% was prepared. Of this suspension, 1 ml. was added to 4 ml. of N/10 HCl and allowed to stand for 30 minutes, when 5 ml. of a 2% sodium cyanide solution was added. The results were read in a photoelectric colorimeter with a green filter. The result obtained with this percentage of ox cells was very near to the 100% reading of the standard for the cyanhaemoglobin method used to estimate haemoglobins in the laboratory, and the ox-cell suspension treated as above was diluted to read within 1% of this standard. A fresh suspension of ox cells was prepared each day. It was realized that this method of standardization only kept the haemoglobin at a constant level, but, since the colour index of the ox bloods employed was fairly constant, the number of red blood corpuscles in the suspensions should have been reasonably constant.

Complement.—Ten minimum haemolytic doses for a 3% suspension of sheep cells sensitized with 5 M.H.D. of rabbit anti-sheep haemolysin were used. This figure was chosen because it would be within the range where minor variations in estimation would have but a small effect and still keep the amount of guinea-pig serum used within reasonable bounds. The only reason that an amount related to sheep cells rather than to ox cells was employed was because complement was being titrated each day for this percentage of sheep cells for routine complement-deviation tests for syphilis, and thus unnecessary work was avoided.

Test.—Serum was inactivated at 56° C. for 30 minutes. Doubling dilutions of serum were made and 0.5 ml. placed in a glass tube, 4 in. × ½ in., to which 0.5 ml. of 2% ox-cell suspension was added. Then 0.5 ml. of 10 M.H.D. of complement was added. The tubes were incubated in a 37° C. water-bath for 15 minutes. A reading was made immediately after removing the rack from the water-bath. The end-point was considered in the tube showing the nearest to 50% haemolysis.

To obtain consistent results it was found necessary either to place the tubes in the water-bath immediately after adding the complement or put them in the refrigerator at 4° C., since haemolysis occurs slowly at room temperature and if this was allowed to continue for long a decrease in the final titre obtained was noted. ("Titre" is used throughout this communication to indicate the reciprocal of the final dilution of the serum before any other reagent is added.)

Experiments

Although there was no reason to believe otherwise it was thought advisable to prove that the haemolysin was absorbed on to the red cells in the absence of complement, as this method was going to be employed later in the investigation to differentiate the antibodies absorbable by sheep cells. The expected results were obtained because the ox cells sensitized with serum from a patient with glandular fever reacted in an identical manner to sheep cells sensitized with rabbit anti-sheep haemolysin.

It has been stated (Bailey and Raffel, 1935) that "the titre of haemolysins in the sera of patients suffering from glandular fever for sheep cells and ox cells were both high." It was therefore decided to carry out experiments to see if this was found to be the case with the sera being investigated (Table I).

TABLE I

TITRE OF HAEMOLYSIN FOR SERA TESTED WITH SHEEP AND OX CELLS

No. of sera	1	2	3	4	5	6	7	8	9	10	11	12
Ox cells ..	320	160	80	80	640	320	40	80	640	1,280	80	160
Sheep cells	10	20	10	10	40	20	40	10	10	20	20	10

The titrations for sheep and ox cells were carried out at the same time and by exactly the same technique, so the results suggest that the titre for ox cells is much higher than the titre for sheep cells in most cases of glandular fever.

In view of the fact that the sheep cells are agglutinated by human sera in a multitude of conditions it was thought possible that these cells might well absorb the haemolysins for ox cells, although the sheep cells themselves were not haemolysed. By following the technique of Dacie (1950) for the absorption of sheep-cell agglutinins by boiled ox cells, but using fresh sheep cells instead of boiled ox cells, no significant reduction in ox-cell haemolysins could be demonstrated. By using the more delicate method of diluting the sera to titre and then absorbing with a large volume of sheep cells and assessing the reduction of the amount of haemolysins obtained by a photo-electric colorimeter it seems as if a very slight absorption does occur.

During the above experiments it was noted that agglutination of the sheep cells in the presence of complement did not appear to absorb the latter substance. Further discussion of this phenomenon is not going to be considered in this paper, as it is to be the substance of a further report. For completeness sera were then tested by the standard method of differential absorption (Dacie, 1950). As was expected, absorption of the serum by guinea-pig kidney made practically no difference to the titre of the ox-cell haemolysins present, while absorption with boiled ox cells invariably removed all of the ox cell haemolysins present in the serum.

The aforementioned experiments showed that haemolysins for ox cells could be determined accurately and consistently and that they were different from sheep cell agglutinins, since even when the sheep-cell agglutinins were completely absorbed ox-cell haemolysins were still present.. It was therefore decided to test for these antibodies in all sera submitted to the laboratory for testing for the presence of heterophile antibodies, for complement-fixing antibodies for encephalitis, mumps, influenza, and lymphocytic choriomeningitis, and also on all sera submitted for investigation for cold agglutinins. This investigation has been carried out for the last 18 months. In addition, during the first few months of the experiment, 2,000 sera submitted for routine testing for syphilis (premarital blood tests) were also examined. Table II shows the number of sera of each titre found in a number of conditions.

These results indicate that in by far the majority of the population the titre of ox-cell haemolysins in the sera when tested by the previously mentioned method is under 10 and it is exceedingly exceptional for it to be over 40.

TABLE II

NO. OF SERA OF EACH TITRE IN CERTAIN CONDITIONS

Condition	No. of Sera Tested	Titre of Ox-cell Haemolysins								
		Under 10	20	40	80	160	320	640	1,280	2,560
Premarital blood tests	2,000	1,998			1				1	
Sera containing cold agglutinins to a titre above 160	26	21	4	1						
Leukaemia	12	10		1	1					
Cancer ..	100	98	1	1						
Infective hepatitis	23	22	1							

Results for Glandular Fever

During the 18 months under review 987 sera were received for testing for heterophile antibodies. Eight hundred and nine of these gave a negative result by the standard method of performing the heterophile antibody test (Dacie, 1950). Of these 809, 799 showed an ox-cell haemolysin titre of less than 20. Of the remaining 178 sera tested, 161 gave a positive Paul–Bunnell reaction, i.e., that the sheep cell agglutinin titre was reduced less than three tubes by the absorption with guinea-pig kidney and that absorption with boiled ox cells reduced the titre of the serum to one-tenth of the original figure. Of these 161 sera, all showed a titre of ox-cell haemolysins of 40 or above. Table III shows the number of sera of each titre.

TABLE III

NO. OF SERA OF EACH TITRE IN 161 SERA

Titre of sera ..	40	80	160	320	640	1,280	2,560
Number of sera	8	51	35	30	25	9	3

The remaining cases gave atypical serological results. Table IV shows the results obtained in a few of these cases.

On a number of these sera weekly estimations of the antibody titres were made in an endeavour to discover whether the ox cell haemolysins and sheep cell agglutinins appeared and regressed at the same rate. No entirely consistent results were obtained, but in by far the majority of the cases examined the titre of the ox-cell haemolysins began to rise earlier and regressed more slowly than the sheep-cell agglutinins.

TABLE IV

Case No.	Date	Sheep Cell Unabsorbed	Absorbed Guinea-pig Kidney	Absorbed Boiled Ox Cells	Ox Cell Haemolysin Titre
1	4/8/51 11/8/51 2/9/51	5,120 2,560 1,280	2,560 2,560 1,280	5,120 2,560 1,280	320 320 160
2	11/12/50 21/12/50	1,280 2,560	1,280 1,280	640 640	20 20
3	21/5/51 9/6/51	1,280 2,560	40 20	40 20	Less than 5 " " 5
4	6/5/51 20/5/51	20 320	20 320	10 40	80 320
5	9/10/51 10/11/51	5,120 2,560	2,560 2,560	1,280 640	320 640
6	7/8/51 15/8/51	20,480 5,260	20,480 5,260	20,480 1,280	5,120 1,280
7	7/6/50 16/6/50	1,280 2,560	160 320	160 320	Less than 5 " " 5

Discussion

The preceding experiments give some information concerning the presence of ox-cell haemolysins in the serum of man, and make some speculation concerning their nature possible. Since ox-cell haemolysins are not absorbed to any extent by sheep cells, and are sometimes absent even when sheep-cell agglutinins are absorbed by boiled ox cells (Table IV, No. 3), and haemolysins for sheep cells are only present at low titres and ox-cell agglutinins almost completely absent, it is therefore suggested that agglutinins of one type and haemolysins of another type are found in the sera of patients suffering from glandular fever. It seems probable that the haemolysins and agglutinins are not merely different manifestations of the same antibody, but different antibodies probably formed in response to different antigens. The absorption of the sera with guinea-pig kidney and boiled ox cells does not help to elucidate the problem, since it shows that the ox-cell haemolysin is not a Forssman antibody and that heating of the ox cells does not destroy the antigenic complex concerned with haemolysis.

These suggestions are contrary to the findings of most previous workers, and bring up the question of whether or not glandular fever with its widely varying clinical manifestations is a single entity or a multiplicity of conditions. The serological results observed in the atypical cases in this investigation give strong support to the latter hypothesis.

Table II shows conclusively that titres of ox-cell haemolysins of over 20 by the technique employed will not frequently be found in the population in general, nor in patients suffering from atypical pneumonia, haemolytic anaemia, leukaemia, infective hepatitis, and carcinoma. Serum sickness is a rare disease these days, but the two cases available only showed a titre of 10 and 20 respectively. The number of sera with titres of over 20 found amongst the 2,000 presumably healthy people (those about to be married) and in the above-mentioned diseases is so low that it is by no means unlikely that these people had previously suffered from glandular fever or had received subinfective doses of the virus, since this is a very common disease in the district from which many of the sera were received. Whether or not this be true, it is not suggested that ox-cell haemolysins are found only in glandular fever, since they appear to be a heterophile antibody and as such must be capable of being produced by many stimulating agents.

Table III shows the titres for cases diagnosed clinically and serologically as classical cases of glandular fever. The figures in this table leave little doubt that cases of glandular fever usually develop ox-cell haemolysins in their sera to a titre many times that found in normal sera, and that the height of the titre is sufficient for its titration to be of help in the diagnosis of this disease. The ox-cell haemolysins usually appear earlier and as, or even more, consistently than the antibodies estimated in the Paul–Bunnell reaction. It would seem therefore that ox-cell haemolysins are a more reliable guide to the presence of glandular fever than sheep cell agglutinins, but that in difficult cases more information can be obtained by doing both tests than either separately.

Conclusions

In all tests where there are antibodies present, but at a low titre in normal serum, it is difficult to decide what titre to consider significant when the titre of these antibodies is raised in disease. This is also the case with ox-cell haemolysins. Naturally the most satisfactory method of diagnosis is to obtain one serum early in the disease and one about a fortnight later and compare the titres. For clinical reasons such a procedure is usually unsatisfactory, as an immediate diagnosis is generally required. If the technique described in this paper is employed it seems certain that a titre of 40 or over can be considered diagnostic of glandular fever in the presence of suitable clinical and haematological findings. A titre of 160 or over is very suggestive of glandular fever without much clinical evidence of the disease. If two or three specimens received at weekly intervals show a titre of less than 20 it is most unlikely that the patient has glandular fever.

It seems as if the estimation of ox-cell haemolysins in the sera is a more valuable procedure in aiding the clinician to diagnose glandular fever than the present Paul–Bunnell reaction even with differential absorption. It also has the advantage of giving a quicker result and is much easier to perform. In difficult cases it is suggested that both the ox-cell haemolysins be estimated and a Paul–Bunnell reaction with differential absorption be carried out.

Summary

A standard technique for estimation of ox-cell haemolysins in human sera is described.

The presence of ox-cell haemolysins to a titre much higher than that found in normal sera is a consistent finding in patients with glandular fever.

It is suggested that an estimate of the ox-cell haemolysins present in the serum is a better aid to the diagnosis of glandular fever than the Paul–Bunnell reaction.

References

Bailey, G. H., and Raffel, S. (1935). *J. clin. Invest.*, **14**, 228.
Browning, C. H. (1931). In "A System of Bacteriology in Relation to Medicine." *Spec. Rep. Ser. med. Res. Coun. Lond.*, vol. 6, p. 501.
Dacie, J. V. (1950). *Practical Haematology*. London.
Davidsohn, I. (1929). *J. Immunol.*, **16**, 259.
—— (1930). Ibid., **18**, 31.
—— (1937). *J. Amer. med. Ass.*, **108**, 289.
—— and Ramsdell, S. G. (1929). *J. Immunol.*, **17**, 365.
Ehrlich, P., and Morgenroth, J. (1899). *Berl. klin. Wschr.*, **36**, 481.
—— —— (1900). Ibid., **37**, 453, 681.
Forssman, J. (1911). *Biochem. Z.*, **37**, 78.
Paul, J. R., and Bunnell, W. W. (1932). *Amer. J. med. Sci.*, **183**, 90.
Stuart, C. A., Welch, H., Cunningham, J., and Burgess, A. M. (1936). *Arch. intern. Med.*, **58**, 512.
Taniguchi, T. (1920). *J. Path. Bact.*, **23**, 368.
—— (1921). Ibid., **24**, 122.
—— (1922). Ibid., **25**, 77.

J. clin. Path. (1952), **5**, 329.

EXTENSIVE MYELOID RESPONSE DURING FOLIC ACID THERAPY IN MEGALOBLASTIC ANAEMIA OF PREGNANCY

BY

GRACE M. RITCHIE

From the Department of Pathology, Stobhill and Western District Hospitals, Glasgow

(RECEIVED FOR PUBLICATION MAY 3, 1952)

Numerous examples of leukaemoid pictures occurring in a variety of conditions have been described in the literature, but I have been unable to trace any case similar to that about to be described.

Case Report

The patient, aged 29 years, para 3, unmarried, and of subnormal intelligence, was first seen in the thirty-fourth week of pregnancy at the hospital antenatal clinic. Previous pregnancies were uneventful and there was no record of serious illnesses. The family history was irrelevant.

The present pregnancy progressed uneventfully until early in the sixth month when she had a severe haemorrhage from a varicose ulcer of the leg. On her first visit to the antenatal clinic on January 15, 1951, she complained of breathlessness, frequency of micturition, and suprapubic pain. On examination she was seen to be of fair nutrition but the skin and mucous membranes were very pale. There was no evidence of icteric pigmentation, koilonychia, glossitis, or of splenic or lymphatic glandular enlargement. No toxaemic manifestations and no abnormalities of heart or lungs were noted. The Wassermann reaction was negative and her blood group was A, Rh negative (no antibodies with anti-D serum). Blood levels were: Hb 7.6 g.%, red blood cells 3.95×10^6/c.mm., colour index 0.69. Examination of a catheter specimen of urine showed oxalate crystals, a few clumps of pus cells, and coliform organisms on culture. She was given a course of sulphamerazine and alkalis pending admission to the obstetrical ward.

After admission on February 2 repeated examination revealed a persistent pyuria ; 30 g. of sulphamerazine, 10.5 g. of chloromycetin, and a subsequent course of sulphatriad were given before the urine became sterile. During the administration of these antibiotics a course of "ferrivenin" was given, in all containing 1,500 mg. of elemental iron. Despite this, the blood values continued to fall.

Two weeks after admission she developed symptoms of an upper respiratory infection, with herpes of the lips, and two episodes of epistaxis. To improve her condition before delivery, she was given two pints of whole blood which raised the Hb to 10.0 g.% and red blood cells to 3.75×10^6/c.mm. Surgical induction was now performed and she was delivered of a healthy girl on February 28 after a labour of seven hours five minutes. Blood loss was minimal. A low-grade intermittent pyrexia persisted until the seventeenth day of the puerperium despite continuous penicillin therapy.

On March 3, after transfer to a medical ward, the Hb had fallen to 4.6 g.% and the red blood cells to 1.9×10^6/c.mm. A white-cell count on March 6 gave 5,400 c.mm. (Table I); platelets, 75,000/c.mm. Peripheral blood films showed moderate polychromasia, marked hypochromia and anisocytosis, a small proportion of macrocytes, extremely scanty platelets, and a few immature white cells. The coagulation time was normal but the bleeding time was prolonged (12 minutes by Duke's method). Sternal marrow examination revealed the presence of megaloblastic erythropoiesis. The serum bilirubin level was 0.4 mg.%. The urinary urobilinogen was excessive. Free hydrochloric acid was present in the gastric contents. Tests for faecal occult blood were negative, and a barium meal and radiograph of the chest were also negative.

Folic-acid therapy, 20 mg. daily, was begun on March 10, as well as two "complevite" tablets three times daily, the latter providing 3 grains ferrous sulphate daily. Within the next 48 hours there was a remarkable outpouring of immature white cells into the peripheral blood (Fig. 1 and Table I), the picture simulating that found in myelogenous leukaemia. These findings persisted for five days only. However, by the sixth day the spleen had become palpable three fingerbreadths below the costal margin although the patient now showed obvious clinical improvement and this had been accompanied by a reticulocyte response of 14% on the fifth day of treatment. The spleen remained palpable for four weeks. Six weeks after the beginning of folic-acid therapy the marrow showed a normal white-cell picture and erythropoiesis

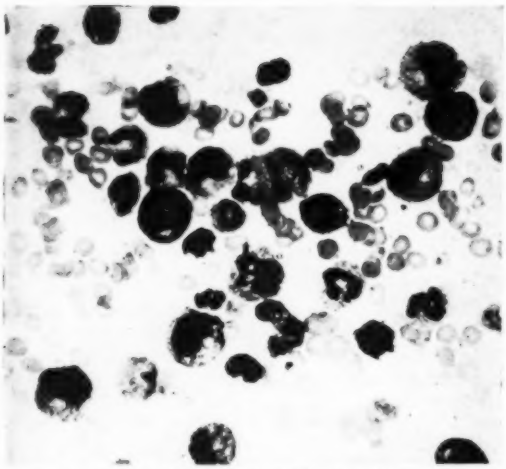


FIG. 1.—Peripheral blood at the height of the leukaemoid phase showing early forms of white cells, × 500.

TABLE I
TOTAL WHITE CELL AND DIFFERENTIAL COUNTS ON PERTINENT DATES

Date	6/3/51	12/3/51	13/3/51	14/3/51	16/3/51	31/5/51
Total/c.mm.	5,400	42,000	21,000	18,400	12,000	4,500
Polymorphonuclears (%) ..	45	40·5	41·0	41·0	37·0	51·0
Metamyelocytes (%)	4	7·5	8·0	13·0	9·0	4·0
Myelocytes (%) ..	1	10·0	5·0	5·0	1·0	—
Premyelocytes (%)	1	4·0	7·0	4·0	2·0	—
Blast cells (%) ..	1	1·5	1·0	0·5	1·0	—
Monocytes (%) ..	9	7·0	7·0	7·5	7·0	8·0
Lymphocytes (%)	34	9·5	15·0	16·0	30·0	37·0
Macropolycytes (%) ..	2	2·5	—	0·5	1·0	—
Normoblasts (%)	1	11·0	10·0	10·5	10·0	—
Megaloblasts (%)	2	4·5	4·0	2·0	—	—
Unclassified (%) ..	—	2·0	2·0	—	2·0	—

was normoblastic. This was again confirmed after a further period of six weeks.

Final blood values before discharge on April 24 were Hb 12.1 g.%, red blood cells 4.5×10^6/c.mm., and white blood cells 6,800/c.mm. (normal differential). No further haematinics have been given and the patient has remained extremely well. Periodic examination to date has shown a completely normal blood picture.

Discussion

Over the past century a large volume of literature has accumulated on the subject of severe anaemias of pregnancy. Most interest, however, has been focused on dyserythropoiesis and relatively little attention has been paid to white-cell abnormalities.

In the so-called pernicious anaemia of pregnancy there may be considerable variation in the state of the white cells. A few published reports mention the occurrence of immature white cells in the peripheral blood (Evans, 1929; Callender, 1944; Moore, Bierbaum, Welch, and Wright, 1945; Wintrobe, 1946). In 1946 Foy, Kondi, and Murray described a case presenting as a macrocytic anaemia, with megaloblastic marrow and free acid in the gastric juice after histamine. In the later stages of the disease the blood became "leukaemic" with 30,000 to 189,000 white blood cells per c.mm., 80–90% of which were myeloblasts. They classified their case as an example of "leukanaemia." In 1948 Gillespie and Ramsay reported a case of pernicious anaemia of pregnancy in which there was such an extreme degree of white cell immaturity as to raise the question of leukaemia; necropsy revealed caseous tuberculosis of the hilar glands and lungs. This association of tuberculosis and a leukaemoid blood picture has already been noted (Custer and Crocker, 1932; Leibowitz, 1938).

It is well known that in megaloblastic anaemias with leucopenia, either liver, vitamin B_{12}, or folic acid are able to produce a rise of white cells into the normal range. However, only in the cases reported by Foy, Kondi, and Murray and by Gillespie and Ramsay has the extent and character of the white-cell response suggested the possibility of leukaemia.

A leukaemoid picture may occur in response to severe infections, including tuberculosis, and also in malignant tumours (Meyer and Rotter, 1942). Of the drugs used in the present case, penicillin alone merits consideration. A case of megaloblastic anaemia has recently been reported by Foy, Kondi, and Hargreaves (1951), in which treatment by penicillin G alone induced a response in no way different from that obtained by potent liver extracts, folic acid, etc. In the present case, however, no improvement in the anaemia and no leucocytosis occurred while penicillin alone was used.

Reference has already been made to the undoubted effect of folic acid on the granular series, and in this case it seems most probable that its effectiveness was enhanced by its administration at a time when a partial spontaneous remission was occurring and when conditions were most favourable for regeneration of all marrow elements. It would, therefore, appear that the case described above presents an exceptionally marked example of the white-cell response which might normally have been expected to occur.

Summary

A case of megaloblastic anaemia of pregnancy, presenting an extensive myeloid response during the course of folic acid therapy, is reported.

The author wishes to express her gratitude to Dr. James McIntosh for permission to publish this case and for much helpful guidance, to Dr. D. McKay Hart for the obstetrical records, and to Mr. Percy Boot for the photomicrograph.

REFERENCES

Callender, S. T. E. (1944). *Quart. J. Med.*, **13**, 75.
Custer, R. P., and Crocker, W. J. (1932). *Folia haemat., Lpz.*, **46**, 359.
Evans, W. (1929). *Lancet*, **1**, 14.
Foy, H., Kondi, A., and Hargreaves, A. (1951). *Brit. med. J.*, **1**, 380.
—— —— Murray (1946). *J. Path. Bact.*, **58**, 157.
Gillespie, M., and Ramsay, A. M. (1948). *Brit. med. J.*, **1**, 828.
Leibowitz, S. (1938). *Arch. Path., Chicago*, **25**, 365.
Meyer, L. M., and Rotter, S. D. (1942). *Amer. J. clin. Path.*, **12**, 218.
Moore, C. V., Bierbaum, O. S., Welch, A. D., and Wright, L. D. (1945). *J. Lab. clin. Med.*, **30**, 1056.
Wintrobe, M. M. (1946). *Clinical Hematology*, 2nd ed., p. 365. London.

J. clin. Path. (1952), **5**, 332.

STUDIES ON THE NORMAL SERUM PANAGGLUTININ ACTIVE AGAINST TRYPSINATED HUMAN ERYTHROCYTES

PART I: THE MECHANISM OF AGGLUTINATION REVERSAL*

BY

THEODORE H. SPAET† AND BETTY W. OSTROM

From the Department of Medicine, Stanford University School of Medicine, San Francisco, California, and the Ziskind Laboratories of the New England Center Hospital, Boston, Massachusetts

(RECEIVED FOR PUBLICATION JULY 15, 1952)

The technique of using trypsinated erythrocytes for the detection of "incomplete" haemagglutinating antibodies was introduced by Morton and Pickles (1947), and has supplied a valuable adjunct in the study of acquired haemolytic anaemias. It is reported to have the advantage of being simple, economical, to give rise to sharp end-points, and produce no prozone when applied to the search for abnormal haemagglutinins (Rosenthal, Dameshek, and Burkhardt, 1951). When trypsinated cells are used in the detection of anti-Rh antibodies the titres obtained tend to be higher than those recorded by the use of albumin techniques and by the indirect Coombs test (Morton and Pickles, 1951; Wiener and Katz, 1951; Wheeler, Luhby, and Scholl, 1950); and the sera of patients with acquired haemolytic anaemia show comparable titres of agglutinins against trypsinated cells in comparison with similar tests with the albumin technique (Rosenthal *et al.*, 1951). Trypsinated cells are especially sensitive in the detection of cold agglutinins (Bouroncle, Dodd, and Wright, 1950) and have been reported useful in the search for lysins (Dacie and de Gruchy, 1951). The major objection to their use has been their tendency to produce false positive results.

Greenwalt (1950) studied a large group of normal male subjects and found that the sera of about 1% of this group agglutinated compatible, trypsinated cells. Five minutes of incubation at 37° C. eliminated about one-third of these "false positives." Morton and Pickles (1951) noted panagglutination against some of their trypsinated cells, which they ascribed to over-trypsinization or to an extended activity of cold agglutinins at room temperature. Rosenthal and Schwartz (1951a, b) performed studies which showed that panagglutination of trypsinated cells by normal compatible serum is a universal but transient phenomenon. These investigators found that in a large number of sera an agglutinin was demonstrable which was active against trypsinated cells either from a compatible donor or from the patient supplying the serum. This agglutinin was active in titres of about 1:16, and had the unique property of becoming ineffective following incubation at room temperature and 37° C. Cells which were agglutinated by centrifugation immediately following exposure to serum dispersed and remained unagglutinable half an hour later if incubation in serum was continued. The speed of this agglutination reversal was proportional to the concentration of serum, proceeding most rapidly in undiluted serum. When the cells which had been previously agglutinated were allowed to become dispersed, these cells were not agglutinated by fresh serum, but the serum in which they had reversed agglutinated fresh cells. The agglutinin and reversal effect were found to be separable. The agglutinin was heat-labile and non-dialysable; the reversal effect was heat-stable and dialysable.

The present study was undertaken to elaborate the mechanism by which a normal panagglutinin clumped trypsinated erythrocytes but lost this ability upon incubation.

Methods and Materials

Blood was obtained from members of the hospital staff, students, and clinic patients who showed no evidence of a haematological disorder.

The blood was obtained in a clean Erlenmeyer flask and defibrinated at room temperature by means of glass beads. After separation of the serum and cells the erythrocytes were trypsinated according to the technique described by Morton and Pickles (1951)

*This work has been supported in part by grants from the American Cancer Society.

†Part of this work was completed during the tenure of a Damon Runyon Clinical Research Fellowship.

with purified cystalline trypsin.* The cells were used within 24 hours of collection.

Agglutinations were performed in standard serological tubes. Equal amounts of the test sera and a 2% suspension of trypsinated cells were mixed at room temperature and agglutination read following a short period of slow centrifugation. Estimations of agglutination were made with the naked eye and graded as follows: 4+, single solid clump; 3+, few large clumps, 2+, numerous clumps of moderate size; 1+, clumping just easily visible; ±, questionable clumping. In reading agglutinations the tubes were tapped gently so that the button of cells was just freed from the bottom of the tube.

In all studies the trypsinated cells were tested against serum from the same blood sample or from the same subject but drawn at a different date; that is, the "auto" technique was used exclusively.

Results

General Observations.—Twenty subjects selected at random all contained the panagglutinin in their serum active against their own trypsinated erythrocytes. As in the studies of Rosenthal and Schwartz (1951a, b), the cells of all agglutinates became completely resuspended upon incubation. This phenomenon is hereafter referred to as "reversal." At room temperature the reversal time was about 30 minutes; at 37° C. it was shortened to about 10 minutes. The observations of Rosenthal and Schwartz (1951a, b) were confirmed in that the reversed cells were not agglutinated by the addition of fresh serum; but serum in which reversal had taken place agglutinated fresh cells.

Effect of Saline Washings on Agglutinated Cells.—Cells were added to serum and centrifuged immediately. The serum was then removed, the agglutinated cell mass was washed three times with normal saline, and was finally suspended in a few drops of normal saline. The cells of such preparations remained strongly clumped for many hours at 37° C., and at room temperature showed little tendency to reverse, sometimes even after standing for 24 hours. A few of these preparations did reverse at room temperature following five hours' incubation. These cells were strongly agglutinated with the addition of fresh serum. Agglutinates which had reversed at 37° C. showed similar behaviour.

In those preparations where reversal had taken place in saline the supernatant fluid was separated. When a fresh agglutinated mass of cells was placed in this supernatant no reversal occurred. These findings suggest that spontaneous elution of the agglutinin from the cells may occur in saline, but does not occur if the concentration of agglutinin is adequately high.

Cells were mixed with serum, spun, and then incubated either at room temperature or at 37° C. until complete agglutination reversal had taken place. They were then washed three times with normal saline and mixed with fresh serum. All such preparations showed 4+ agglutination. When this same experiment was repeated, except that the washed cells were replaced into the original serum in which they had previously reversed, again they showed 4+ agglutination. It is thus evident that neither the cells nor the serum underwent any irreversible change following agglutination reversal. It was noted in addition that when a tube containing cells which had undergone agglutination reversal was placed into the refrigerator the normal cold agglutination was evident with unimpaired activity.

Effect of Repeated Exposure of Serum to Fresh Cells.—Sera were divided into three aliquots. One aliquot was treated with fresh, packed cells seven times, the cells having been centrifuged and removed immediately following mixing. The second aliquot was likewise exposed to seven changes of packed cells, but in each case the cells were allowed to undergo agglutination reversal before removal. The third aliquot served as a control, and was incubated for the same period as the others. Following treatment the three aliquots were titred for their agglutinin activity. A typical titration of such sera is shown in Table I. It can be seen that immediate removal of the cells causes a definite loss of agglutinin activity, whereas allowing the agglutinate to reverse in the serum does not cause a reduction in the agglutinin titre. These findings suggest that reversal is associated with elution of the agglutinin off the cell surface.

TABLE I

EFFECT OF REPEATED EXPOSURE OF SERUM TO FRESH TRYPSINATED CELLS

	1:2	1:4	1:8	1:16	1:32	1:64
Trypsinated cells against normal serum ..	4+	4+	3+	1+	0	0
Trypsinated cells against absorbed serum ..	2+	1+	0	0	0	0
Trypsinated cells against reversed serum ..	4+	4+	3+	1+	0	0

Effect of Sodium Cyanide on Agglutination.—Cells and serum were centrifuged immediately after mixing. The agglutination was read, and one drop of sodium cyanide dissolved in normal saline was added. Sodium cyanide in concentrations of 0.1% to 0.0001% was used, In dilutions of up to 1:500,000 and at a *p*H range of 5 to 9 sodium cyanide caused almost immediate reversal of strongly agglutinated cells. The cyanide effect was evident on agglutinated cells suspended either in serum or in saline. When cells which had been subjected to agglutination reversal with cyanide were washed three times with normal saline they were again agglutinated as strongly as before treatment, following the addition of fresh serum.

Sodium cyanide in concentrations of up to 1% did not affect the agglutination of A cells by anti-A serum, B cells by anti-B serum, or D+ cells by anti-D serum. Preliminary studies appear to indicate that the titre of cold and warm auto-agglutinins is reduced by the action of sodium cyanide.

*Supplied by the Armour Laboratories, Chicago, Illinois.

The means by which cyanide induced reversal was studied as follows. Packed cells were added to serum and removed repeatedly until the serum showed a definite loss of agglutinating ability. The agglutinated cells were washed three times with normal saline and pooled. To these pooled cells was added saline to bring the volume to that of the original absorbed serum. Three drops of 0.1% sodium cyanide were then added to this suspension, and the dispersed cells removed by centrifugation. The supernatant fluid was then dialysed in a Visking bag for 24 hours against normal saline buffered to *p*H 7.4 with one-tenth its volume of Sørenson's phosphate buffer. Five 200-ml. changes of dialysing fluid were used. The resulting preparation agglutinated fresh cells, and there was no reversal at room temperature. Evidently sodium cyanide reverses the agglutination by causing elution of the agglutinin.

The effect of other known enzyme inhibitors and activators was studied. The following agents did not affect the rate of agglutination reversal in serum: saturation with carbon monoxide, 0.1% sodium azide, 25% urethane, 10% formaldehyde, 0.13% acetic acid, 0.1% ammonium hydroxide, 0.1% nitrogen mustard; 2 mg.% creatinine, 20 mg.% urea, 5 mg.% uric acid, 0.1% solutions of d. alamine, asparagine, glycine, and tyrosine; 0.1% solutions of the cations Mg^{++}, Al^{+++}, Fe^{++}, Fe^{+++}, Hg^{++}, Ba^{++}, Ca^{++}, Sr^{++}, Cu^{++}; and 0.1% solutions of the anions PO_4^{---}, I^-, Br^-, SO_4^{--}, acetate.

Mode of Action of the Reversor Agent.—Serum was heated at 60° C. for two hours. Such serum had no agglutinating activity, but caused reversal of washed agglutinated cells with normal activity, as has been previously reported (Rosenthal and Schwartz, 1951b.) Urine was also found to be an active reversing agent, although some urines had to be boiled to remove a slight tendency to cause non-specific agglutination. Of 2% cell suspension, 0.2 ml., and 0.2 ml. of serum were mixed, then centrifuged immediately. The agglutinated cells were then washed three times in saline and suspended in 0.2 ml. of normal serum, heat-inactivated serum, urine (neutralized to *p*H 7) or normal saline. Periodic readings were made to determine the persistence of agglutination. It can be seen from Table II that the reversal induced by urine is actually more rapid than that by serum.

Cells from 0.2 ml. of 2% suspension were incubated with 0.2 ml. of urine or inactivated serum for one hour. The urine or serum was then removed, and 0.2 ml. of fresh serum was added. The mixture was centrifuged immediately and read for agglutination. These preparations were all negative.

In every case washing the cells three times with normal saline restored their ability to agglutinate in fresh serum.

These findings suggest that the reversor agent is adsorbed on to the cell surface in loose combination, and that its presence renders the cell refractory to the action of the agglutinin.

TABLE II

EFFECT OF URINE AND SERUM ON THE SPEED OF AGGLUTINATION REVERSAL

	0	5 min.	15 min.	30 min.	1 hour	2 hours
Agglutinate in normal serum	4+	4+	1+	0	0	0
Agglutinate in saline ..	4+	4+	4+	4+	4+	3+
Agglutinate in heated serum	4+	2+	0	0	0	0
Agglutinate in saline: urine added	4+	0	0	0	0	0

The Effect of Increased Trypsinization.—Cells were trypsinated as before except that the concentration of trypsin was increased and the time of incubation was prolonged. Neither the rate of reversal with incubation nor the susceptibility to reversal by sodium cyanide was affected by trypsinization with enzyme solutions up to 1% incubated for up to four hours. Within the limits of the present study it does not appear that over-trypsinization universally results in "false positive" panagglutination. There is evidently a considerable degree of latitude in this test.

Discussion

On the basis of the findings reported herein it is possible to postulate the mechanism of agglutination and agglutination-reversal when trypsinated erythrocytes are exposed to compatible serum and incubated. The serum normally contains an agglutinin which is active against trypsinated cells. The rate of combination of this agglutinin with the cell surface is rapid, but its union is loose and unstable. Also present in the serum is another agent which competes with the agglutinin for the cell surface. Its combination rate is slower, but its affinity is greater than that of the agglutinin. Thus the initial agglutination follows the rapid adsorption of the agglutinin on to the erythrocyte, and the subsequent reversal appears to follow displacement of the agglutinin by the more sluggish but more tenacious reversor. That the reversor is actually adsorbed on to the cell's surface is shown by the production of inagglutinable cells when there is previous incubation of cells with reversor. However, even the reversor must be on the cell surface in rather loose combination, as it can easily be removed simply by washing the cells in normal saline. The present phenomenon does not appear to be analogous to the adsorption-elution sequence described by Hirst (1948) in relation to erythrocytes exposed to certain viruses. The cells in those studies suffered permanent loss of agglutinability by the virus following elution.

Sodium cyanide mimics the action of the naturally present reversor substance by causing

elution of the agglutinin, although it is much more potent than the natural agent. The minute concentrations of cyanide needed to cause elution suggest that an enzyme system may be involved. It is certainly not related to residual trypsin activity, as the phenomena described are not affected by repeated washings. Moreover, trypsin activity is not affected by cyanide (Calandra *et al.*, 1951).

The nature of the alterations on the erythrocyte surface produced by the action of trypsin is still a matter of conjecture. The ability of bacterial products to render red cells panagglutinable by normal sera has long been known (Thomsen, 1927; Friedenreich, 1928). Burnet, McCrea, and Stone (1946) have shown that viruses possess the same ability, and that erythrocytes modified by these agents show altered antigenic properties (Burnet and Anderson, 1947). One of the substances which was effective in inducing these changes was found to be an extract of *Vibrio cholerae*, and it was discovered by Pickles (1946) that erythrocytes treated with these extracts were susceptible to agglutination by "incomplete" anti-Rh antibodies. Chu and Coombs (1947) and Chu (1948) extended this finding to include a wide variety of bacteria and viruses showing that these organisms all had the ability to modify erythrocytes in such a way that there was susceptibility to panagglutination, loss of virus preceptors, and increased sensitivity to "incomplete anti-Rh." The various organisms had differing and uncorrelated potencies in effecting one or another of these modifications. Most recently Stewart and Meenan (1951) have shown that the action of trypsin differs from that of vibrio extracts and virus. Trypsin does not destroy the virus receptors in concentrations sufficient to produce a high degree of sensitivity to anti-Rh antibodies. In contrast trypsinized cells are much less susceptible to "T" agglutination than are virus and vibrio-treated cells. Treatment of erythrocytes with periodate also renders them panagglutinable by normal sera, but this agent destroys the ability of the cells to react with anti-Rh along with the virus receptors, and produces an antigenic modification which differs from that caused by the previously mentioned agents (Stewart and Meenan, 1951; Stewart, 1949; Moskowitz and Treffers, 1950). It thus appears as if the modification of the erythrocyte produced by viruses and bacterial extracts is comparable to that produced by trypsin, but is by no means identical.

Ponder (1951) has studied the physical properties of erythrocytes treated with trypsin, and has been able to demonstrate minor alterations, including increased volume and mechanical fragility as well as reduced electrophoretic mobility. Other discussions as to the nature of the surface alteration in these cells have been hypothetical.

Studies are now in progress which are attempting to define the nature of the normal serum agglutinin active against trypsinated erythrocytes, and the agent which causes reversal of this agglutination. It is also hoped to determine whether any relationship exists between this normal agglutinin and the agglutinins found abnormally in acquired haemolytic anaemia.

Summary

Studies were performed in which the agglutination of trypsinated human erythrocytes by normal human serum and the reversal of this agglutination upon incubation were analysed.

Cells which had agglutinated and reversed regained their agglutinability in fresh serum and in the original serum after having been washed with saline. Reversal was inhibited in saline.

It was shown that reversal was accompanied by elution of the agglutinin, and this occurred upon incubation or upon the addition of sodium cyanide.

Heated serum and urine both contain reversor agent free of agglutinin. Cells incubated with these substances are rendered inagglutinable. Agglutinability can be restored by washing.

It is concluded that agglutinin and reversor are in competition for the red cell surface. The affinity of agglutinin is weak, but the speed of reaction is rapid. The affinity of reversor is stronger, but its speed of reaction is considerably slower.

The authors wish to thank Drs. Martin C. Rosenthal and Rose Paine for their valuable help.

References

Bouroncle, B. A., Dodd, M. C., and Wright, C. (1950). *J. Lab. clin. Med.*, **36**, 801.
Burnet, F. M., and Anderson, S. G. (1947). *Aust. J. exp. Biol. med. Sci.*, **25**, 213.
—— McCrea, J. F., and Stone, J. D. (1946). *Brit. J. exp. Path.*, **27**, 228.
Calandra, J. C., Hardt, L. L., and Stanish, E. S. (1951). *Gastroenterology*, **19**, 564.
Chu, C. M. (1948). *Nature, Lond.*, **161**, 606.
—— and Coombs, R. R. A. (1947). *Lancet*, **1**, 484.
Dacie, J. V., and de Gruchy, G. C. (1951). *Journal of Clinical Pathology*, **4**, 253.
Friedenreich, V. (1928). *C. R. Soc. Biol., Paris*, **98**, 894.
Greenwalt, T. J. (1950). *J. Lab. clin. Med.*, **36**, 828.
Hirst, G. K. (1948). *J. exp. Med.*, **87**, 301.
Morton, J. A., and Pickles, M. M. (1947). *Nature, Lond.*, **159**, 779.
—— —— (1951). *Journal of Clinical Pathology*, **4**, 189.
Moskowitz, M., and Treffers, H. P. (1950). *Science*, **111**, 717.
Pickles, M. M. (1946). *Nature, Lond.*, **158**, 880.
Ponder, E. (1951). *Blood*, **6**, 350.
Rosenthal, M. C., Dameshek, W., and Burkhardt, R. (1951). *Amer. J. clin. Path.*, **21**, 635.
Rosenthal, M., and Schwartz, L. (1951a). *Fed. Proc.*, **10**, 332.
—— —— (1951b). *Proc. Soc. exp. Biol., N.Y.*, **76**, 635.
Stewart, F. S. (1949). *J. Path. Bact.*, **61**, 456.
—— and Meenan, P. N. (1951). *Irish J. med. Sci.*, **6**, 6th ser., No. 311, 541.
Thomsen, O. (1927). *Z. ImmunForsch.*, **52**, 85.
Wheeler, W. E., Luhby, A. L., and Scholl, M. L. L. (1950). *J. Immunol.*, **65**, 39.
Wiener, A. S., and Katz, L. (1951). Ibid., **66**, 51.

J. clin. Path. (1952), **5**, 336.

A CASE OF THE SCHÖNLEIN-HENOCH SYNDROME WITH MYOCARDIAL NECROSIS

BY

M. A. LECUTIER

From the Department of Pathology, The Royal Infirmary, Derby

(RECEIVED FOR PUBLICATION APRIL 16, 1952)

The purpuras of Henoch and Schönlein have usually been described together under the term "anaphylactoid purpura." It is only in recent times, however, that papers on their pathology have appeared in which a definite lesion, which is common to both conditions, is described. This consists of an arteriolitis which, in severe cases, is necrotizing. It is now reasonably well established that the Schönlein-Henoch syndrome is due to a hypersensitivity reaction in which either a bacterial or a non-bacterial antigen, for example, an article of food, may be concerned (Gairdner, 1948).

It is the purpose of this communication to describe a case in which there was an opportunity to study the pathology after death. The case is of particular interest because much attention is being paid at present to the collagen diseases and because a cardiac lesion was found which apparently has not been described before in this syndrome.

Case Report

A. K. H., a boy of 11 years of age, was admitted to hospital complaining of a haemorrhagic rash and also of abdominal pain with vomiting. He had been perfectly well until six days before admission, when he had developed an exanthem on both legs, which in the first place consisted of vesicles. Two days before admission the joints had begun to swell, first the knees and then the elbows. There was nothing of note in the past history. On examination there were multiple small haemorrhagic macules on both legs, feet, and hands, and also many small discrete haemorrhagic vesicles on both legs and feet. Diffuse joint swellings were a feature, affecting both elbows, wrists, hands, and knees. The fauces were injected but the heart and lungs revealed no abnormal physical signs. The abdomen and central nervous system revealed no abnormality. The blood pressure was 142/98 and the optic fundi presented narrow arterioles only. Two days later fresh petechiae appeared on the buttocks, and the next day a soft systolic apical murmur was noticed for the first time. Simultaneously several large purpuric patches appeared on the hands and feet, blood was passed per rectum, and the urine was noticed to be smoky. The blood pressure rose to 145/105 and the blood urea was 200 mg.%. Penicillin therapy was instituted and it cleared the throat of haemolytic streptococci. Thereafter the patient's condition slowly deteriorated and he died approximately one month after admission.

Numerous investigations were performed. Albuminuria was present, together with varying numbers of casts, pus cells, and red cells, from the day of admission. Haemolytic streptococci were isolated from a throat swab two days after admission. The blood count on admission showed no anaemia but there was marked polymorphonuclear leucocytosis. Anaemia gradually developed and the leucocytosis persisted. The platelet count was never below normal. Bleeding and clotting times were found to be normal on several occasions. The cerebrospinal fluid and faeces likewise were normal on repeated examination.

Necropsy Report

Externally there was moderate pallor and anasarca. Several dried haemorrhagic lesions were seen on the fingers and toes and one on the left forearm.

The brain presented generalized oedema of moderate degree. The lungs were oedematous and rubbery in consistency. The pericardium contained a large volume of clear, straw-coloured fluid. The heart was a little dilated and the valves were normal; the myocardium was rather pale and there were a few scattered, small, hard nodules of irregular outline in the wall of the left ventricle; the coronary arteries appeared normal. The liver presented a mild degree of passive congestion. Both kidneys were large and pale with broad, pouting cortices. The mucosa of the bladder was haemorrhagic but otherwise normal. The small intestine presented several discrete haemorrhagic patches in its wall and the terminal 18 inches of the ileum were thus uniformly affected. The peritoneal cavity contained a fairly large volume of clear, straw-coloured fluid. None of the large arteries appeared abnormal in any way and all other organs and parts appeared normal.

Histologically, sections from the spleen, the

skin, the bladder, the ileum, the lungs, the kidneys, and the liver presented a periarteriolar infiltration with lymphocytes, histiocytes, polymorphs, and eosinophils, and, moreover, the arteriolar walls were themselves infiltrated by these cells, and in many cases there was in addition fibrinoid necrosis of the walls. In the case of the bladder and ileum, the lumina of some of these necrotic vessels were plugged with hyaline thrombus. As regards the heart, although several blocks and serial sections were examined, only a very few arterioles showed any sign of involvement and these were very small and showed no evidence of superadded thrombosis. The capillaries presented a similar perivascular infiltration in the case of kidneys, the liver and the skin, and there was patchy necrosis of the glomerular tufts of the kidney. Apart from the vascular lesions, the myocardium presented several areas of necrosis with calcification and some of these were extensive; they corresponded to the hard nodules found at necropsy; none was found to be related to any involved arterioles, none of which in any case showed any evidence of thrombosis. In addition, several necrotic areas were seen without calcification, but these were not as large as the calcified ones. Surrounding each lesion there was a mild degree of infiltration with small round cells only (Fig. 1). The kidneys presented the appearances of a subacute nephritis (Ellis type 1) with typical epithelial crescents; moreover the stroma showed a mild degree of patchy infiltration with lymphocytes, eosinophils, and polymorphs. The liver showed a moderate degree of fatty degeneration. The appearances in the lungs were consistent with those of so-called uraemic pulmonary exudate (Doniach, 1947).

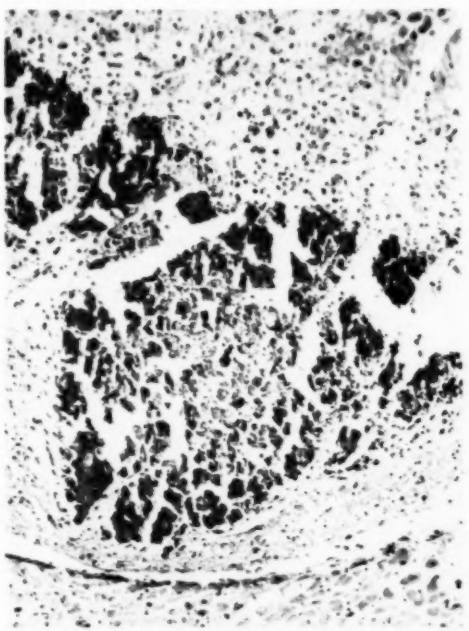


FIG. 1.—Photomicrograph (× 80) of area of myocardial necrosis with calcification. There is only a mild degree of surrounding cellular infiltration, the cells being practically all lymphocytes. Stained by haematoxylin and eosin.

Z

Discussion

In all essentials the case described is typical: clinically in that the three main symptoms, an exanthem, gastro-intestinal upset, and joint lesions, were present; aetiologically in that there was an original haemolytic streptococcal infection as a result of which a hypersensitivity mechanism probably operated; and pathologically in that the basic lesion was an acute arteriolitis with perivascular infiltration (Spiegel, 1936; Miller and Daley, 1946; Gairdner, 1948).

There is little information in the literature concerning the histological appearances of the lesions in the gut. The typical lesion of necrotizing arteriolitis was found in this case and thrombosis was superadded (Fig. 2). Homb (1946) described a case in which typical lesions were found in the gut.

However, the areas of myocardial necrosis with calcification constitute the most interesting feature of this case. Apparently such a lesion has not been previously described in the Schönlein–Henoch syndrome and its pathogenesis is a matter of doubt. There would appear to be three lines of approach in attempting an explanation: (1) The lesion may be the result of ischaemia, resulting from necrotizing arteriolitis with thrombosis. (2) The lesion may represent a modified Aschoff body. (3) The lesion may be the result of the same mechanism as that producing the vascular lesions.

With regard to the first possible explanation, although this appears to be the most reasonable one, no practical support was forthcoming because no arteriolitis with thrombosis was found in the myocardium although several blocks and several sections were examined. Only a very few small arterioles showed any evidence of involvement in all these sections, none showed thrombosis, and none was related to the areas of necrosis described; moreover these areas of necrosis were large, such as could only be produced by blockage of fairly large vessels and would hardly escape detection in serial sections. The theory of ischaemia is therefore unacceptable.

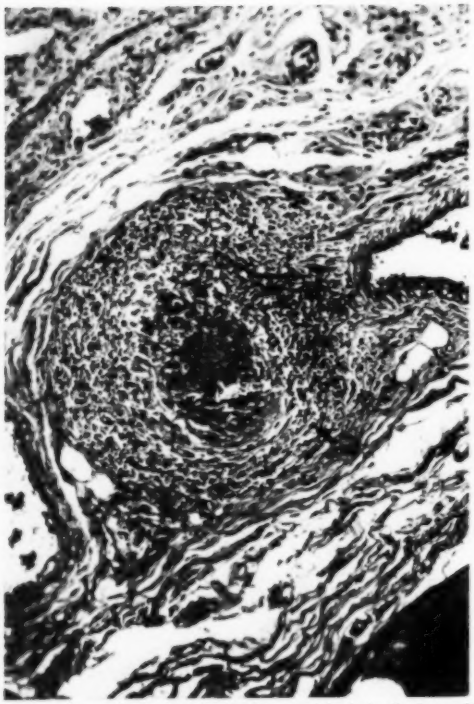


FIG. 2.—Photomicrograph (× 80) of an arteriolar lesion in the small intestine, showing heavy cellular infiltration of the vessel wall, the lumen being plugged by a thrombus. Stained by haematoxylin and eosin.

With regard to the second suggested explanation, it must be borne in mind that polyarteritis nodosa, and presumably its variant the Schönlein–Henoch syndrome, is closely allied to acute rheumatism. Thus both conditions are in all probability allergic diseases (Gairdner, 1948; Rich and Gregory, 1943), the vascular lesions of each are very similar, and fibrinoid necrosis occurs in each. That polyarteritis nodosa and rheumatic fever are closely allied is shown by Friedberg and Gross (1934), who described in detail four cases of polyarteritis nodosa associated with rheumatic fever. They believe in fact that rheumatic fever is a common cause of polyarteritis nodosa. One might therefore postulate that the necrotic myocardial lesion described represents a modified Aschoff body. The virtual absence of any surrounding inflammatory cells in the case of the lesion in the present case indicates, however, that the lesion is certainly not identical with the Aschoff body, but it may be argued that it represents the first stage of an Aschoff body before there has been adequate time for a cellular reaction to occur. The large size of the lesions, together with the calcification make this explanation unlikely. That the lesion is a modified Aschoff body is therefore unacceptable.

As the first two explanations are unacceptable, although initially they appeared the most logical ones, one must accept the third explanation in the absence of a better. Fundamentally there would appear to be no reason why an antigen-antibody reaction which affects the arterioles and capillaries should not also affect the myocardium. If this is the correct explanation, it would, however, be expected that the lesion would have been described before. It must be concluded that some unknown additional factor is concerned and future observations may bring this to light. That an additional factor may be concerned in antigen-antibody reactions is shown by Ahlström (1936) who produced diffuse glomerulitis in rabbits sensitized to horse serum only after an injection of a small quantity of *Staphylococcus* toxin.

Finally one should consider briefly the relation of the Schönlein–Henoch syndrome to polyarteritis nodosa. Undoubtedly the lesions in these two conditions are essentially the same. Moreover the clinical resemblance between the two conditions is impressive; thus skin, joint, and intestinal lesions are all well described in polyarteritis nodosa (Spiegel, 1936; Miller and Daley, 1946). Gairdner (1948) stresses the fact that capillaries are involved in the Schönlein–Henoch syndrome and arteries in polyarteritis nodosa, arteriolar lesions being common to both. Certainly in this case arterioles and capillaries only were involved, but this cannot be considered a fundamental difference between the two syndromes. Spiegel (1936) refers to capillary lesions occurring in polyarteritis nodosa, and, moreover, the frequent occurrence of nephritis in polyarteritis nodosa is evidence of frequent capillary involvement. It is suggested therefore that the two syndromes constitute the same condition, and that whether the arteries or capillaries are involved depends on variations in the conditions of pathogenesis operating in any particular case.

Grateful acknowledgment is made to Dr. D. V. Hubble, under whose care the patient was admitted, for access to the clinical details, and to Dr. G. R. Osborn for his helpful advice.

REFERENCES

Ahlström, C. G. (1936). *Acta path. microbiol. scand. Suppl.* **29**.
Quoted in Hadfield, G., and Garrod, L. P. (1947). *Recent Advances in Pathology*, 5th ed. London.
Doniach, I. (1947). *Amer. J. Roentgenol.*, **58**, 620.
Friedberg, C. K., and Gross, L. (1934). *Arch. intern. Med.*, **54**, 170.
Gairdner, D. (1948). *Quart. J. Med.*, **17**, 95.
Homb, A. (1946). *Acta med. scand.*, **124**, 404.
Miller, H. G., and Daley, R. (1946). *Quart. J. Med.*, n.s., **15**, 255.
Rich, A. R., and Gregory, J. E. (1943). *Bull. Johns Hopk. Hosp.*, **73**, 239.
Spiegel, R. (1936). *Arch. intern. Med.*, **58**, 993.

J. clin. Path. (1952), 5, 339.

THE HISTOGENESIS OF FOUR CASES OF ANGIOMYXOMA OF THE AURICLE

BY

C. RAEBURN

From the Area Pathological Laboratory, Whipps Cross Hospital, Essex

(RECEIVED FOR PUBLICATION MAY 22, 1952)

Many individual primary tumours of the heart have now been reported and there are two general reviews in the English literature, those of Yater (1931) and Prichard (1951), and also an extensive monograph in French by Mahaim (1945). The addition of a few more would be of little interest, but the study of 10 benign tumours, collected from some 19,000 necropsies performed by the staff of a central histological laboratory, has yielded facts of importance in the aetiology and designation of these lesions. Four of these tumours were angiomyxomas arising in the left auricle and they alone are considered in this paper.

Technique

Portions of tissue were fixed in formol saline and embedded in paraffin wax. The following stains and histochemical methods were employed; haematoxylin and eosin, iron haematoxylin and Van Gieson, Heidenhain's iron haematoxylin, Mallory's phosphotungstic acid haematoxylin, and Weigert's stain for elastic fibres. Mucin was demonstrated by Southgate's method, by toluidine blue, and by the periodic-acid-Schiff reaction (P.A.S.). The last two methods were also used after prior treatment of the sections with hyaluronidase. Best's method was employed as well as P.A.S. to demonstrate glycogen. Iron was demonstrated by the method of Perls. Frozen sections of one myxoma were stained by Sudan III for fat.

Case Reports

Positive clinical findings were available for one case; the remaining three were apparently free of cardiac symptoms. In two instances the myxoma was undoubtedly responsible for death.

Case 1 (406/51).—A man aged 68 complained of attacks of shortness of breath, cough, pain in the chest and swelling of the legs over a period of a year. The physical signs were those of intermittent congestive heart failure with bilateral pleural effusions. There were no cardiac murmurs but auricular flutter and partial heart block occurred at intervals (Fig. 1). After many relapses and recoveries he died suddenly during an attack of congestive heart failure. The body was that of a well-nourished but oedematous man. The general visceral findings may be summarized as those of prolonged right heart failure in every way comparable with those due to mitral stenosis. There were small pleural effusions and the liver and spleen were enlarged. The lungs contained multiple infarcts, and organizing thrombi were present in many of the intrapulmonary branches of the pulmonary artery. The right ventricle and the left auricle were greatly dilated and the latter contained a pear-shaped mass ($4.5 \times 3.8 \times 3.5$ cm.) which was attached by a short, flattened pedicle to the lower border of the fossa ovalis and depended into the mitral orifice in such a manner as to maintain the valve cusps in the half-open position (Fig. 2). The mass was of elastic consistency and the external surface, mainly smooth but with some areas of velvety texture, was deep red with a mottling of yellow. The

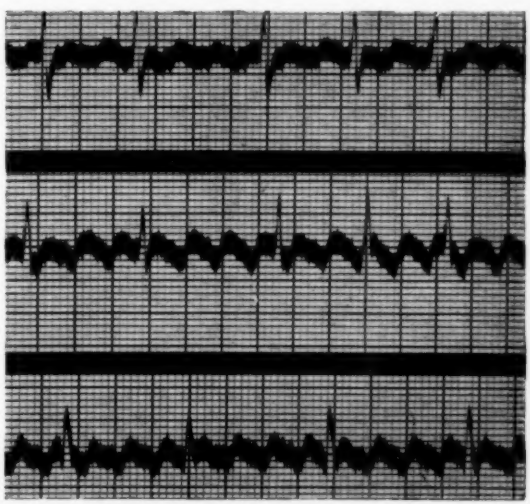


FIG. 1.—Electrocardiogram showing auricular flutter and partial heart block due to myxoma of left auricle.

FIG. 2.—Cross section of myxoma of left auricle. The auricle is hypertrophied, the mitral cusp and chordae tendineae are slightly thickened.

cut surface was composed of soft, yellow tissue with haemorrhagic areas. The mitral valve cusps and the chordae tendineae were slightly and uniformly thickened, but without distortion or fusion of the margin of the cusps, and the left auricle was hypertrophied to a thickness of 0.6 cm. but there was no suggestion of endocarditis. Fragments of ante-mortem thrombus were present in both auricular appendices.

Histology.—The surface of the tumour was covered by endothelium and there were occasional groups of short blunt villi corresponding to the gross areas of velvety texture. Beneath the endothelium was a fibro-elastic lamina with numerous breaches of continuity where the elastic fibres ended in hyalinized collagen bundles or had become separated and ultimately lost in structureless areas. Towards the pedicle the elastic lamina was intact and it fused with the superficial fibres of the elastica of the auricle, which thus divided to enclose the tumour (Fig. 3). Formed blood vessels were confined to the pedicle but capillaries and large vascular spaces lined by endothelium were plentiful. Stromal haemorrhages were numerous and iron pigment was diffusely scattered. The bulk of the tumour was composed of mucinous material in which collagen and elastic fibres ramified irregularly. Lying in groups, strands, and in branching formations in the myxomatous stroma were the lepidic* cells characteristic of auricular myxoma (Fig. 4). Many of these cells resembled swollen and rounded fibroblasts which were grouped into slender solid cords, but in some strands a lumen, in which red cells were occasionally present, had appeared (Fig. 4). Every transition could be found from solid cords through primitive vascular channels to large, irregular vascular spaces lined in part by cubical or fusiform cells and in part by flattened endothelium. This was a classical "myxoma" but its essentially angiomatous nature was readily apparent. When sections were treated with toluidine blue small areas of metachromatic staining appeared at random in the matrix and particularly in the region of the large vessels in and near the pedicle. This metachromatic substance was removed by treatment with hyaluronidase. The matrix as a whole stained well by Southgate's mucicarmine and the P.A.S. technique and this mucin was not digested by hyaluronidase. Glycogen and fat were absent.

Case 2 (1076/37).—A woman aged 78, with no history of cardiac or pulmonary disease, died from gangrene of the rectum. At necropsy a pear-shaped, slightly lobulated mass ($5 \times 3 \times 2.5$ cm.) was found arising from the lower border of the fossa ovalis and depending through the mitral orifice by which it was slightly constricted. The tumour was of rubbery con-

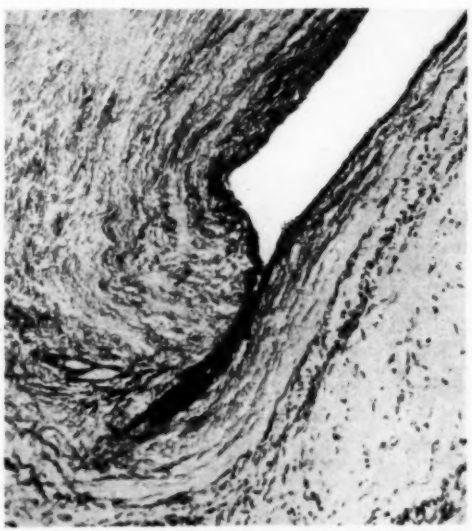


FIG. 3.—The superficial elastic fibres of the auricle (left) are reflected on to the surface of the myxoma. (Weigert's elastic stain, × 142.)

* The term "lepidic" means pertaining to a lining membrane. It has for long been applied to the principal cells of these tumours and is justified as there is a continual evolution of solid cords of cells and eventually of vascular spaces. The fibroblast is regarded as the precursor of the lepidic cell which is the precursor of the endothelial cell.

sistency and mottled red and yellow on both the external and the cut surfaces. The cusps of the mitral valve and the chordae tendineae were slightly and uniformly thickened but there was no evidence of endocarditis.

Histology.—The tumour was covered by endothelium and beneath this there was a condensation of elastic fibres which increased in thickness towards the base and became continuous with the superficial fibres of the auricular elastica. The main bulk of the tumour was composed of structureless mucinous material in which delicate elastic and collagenous fibres ramified. In degenerate areas collagen and elastic fibres were heavily impregnated with iron (Fig. 5). Scattered in the gelatinous matrix were groups and strands of lepidic cells undergoing vascular differentiation and there were a few formed vessels with muscle fibres and an elastic lamina in the region of the pedicle. Large vascular spaces in the matrix were lined by flattened endothelium, with interpolated groups of fibroblast-like cells often several deep, and contained by a single collagen strand. The vasoformative elements were clearly the important histological component. The subjacent auricular elastic tissue was atrophied but the auricular muscle was hypertrophied. The mucinous ground substance took all the mucin stains diffusely and was focally metachromatic with toluidine blue. Hyaluronidase acted only on these metachromatic areas.

Case 3 (526/38).—A man aged 73, with no history of any thoracic disability or pain, died from oedema of the lungs following prostatectomy. At necropsy an elongated, pear-shaped tumour, similar to those already described and measuring $7.5 \times 5 \times 4$ cm., was found to arise from the posterior border of the fossa ovalis and to extend through the mitral valve, by which it was slightly constricted, to fill most of the cavity of the left ventricle (Fig. 6). The mitral valve cusps were normal but the left ventricle was hypertrophied to a thickness of 1.8 cm.

FIG. 5.—Iron impregnation of collagen bundles in auricular myxoma. (Perls' method, × 160.)

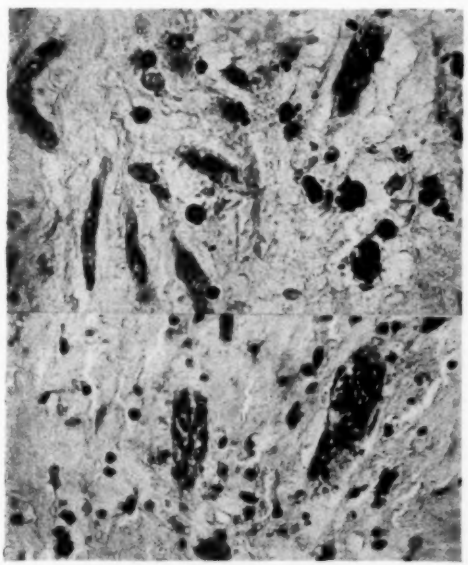


FIG. 4.—Vasoformative lepidic cells in auricular myxoma. (Haematoxylin and eosin. Top, × 340, below, × 200.)

Histology.—The mass was partly covered by endothelium and partly by a thin lamina of ante-mortem thrombus. Lepidic cells, a few of which contained fine particles of glycogen, were scattered singly and in vasoformative groups in a mucinous stroma containing collagen and elastic fibres and much iron pigment. The tumour vessels were mainly large, endothelium-lined spaces, each bounded by a single collagen fibre but towards the base there were formed vessels with a muscular coat and an elastic lamina. Elastic fibres were condensed peripherally in the region of the angle between the tumour and the auricle and the superficial fibres of the auricular elastica were continued into the periphery of the tumour until they vanished into areas of necrosis. Beneath the base of the tumour the elastic tissue of the auricle had atrophied and foci of lepidic cells were mingled with the auricular muscle fibres.

Case 4 (1466/40).—This lesion was an incidental post-mortem finding in a man aged 69 dying of bronchopneumonia associated with a buccal carci-

FIG. 6.—A large myxoma of the left auricle which passes through the mitral valve and fills the cavity of the left ventricle.

noma. A slightly lobulated, firm, dark red nodule 1.5 cm. in diameter projected into the left auricle immediately below the lower border of the fossa ovalis. The cut surface was composed of red, spongy tissue enclosed by a fibrous capsule 0.1 cm. in thickness.

Histology.—The tumour was a capillary and cavernous haemangioma. The smaller vascular spaces were enclosed by swollen endothelial cells, the larger having, in addition, a wall composed of delicate, intertwined collagen fibres separated in places by a slightly mucinous matrix, and there were occasional small groups of cells of lepidic appearance. Iron pigment was scattered in coarse, granular aggregates and collagen and elastic fibres were often impregnated with iron. There were several fragments of bone (Figs. 7 and 8). Peripherally condensed elastic fibres could not be traced into continuity with those of the auricle as the auricular elastic tissue beneath and lateral to the angioma was deficient, either primarily, as a congenital hypoplasia, or secondarily as a pressure atrophy. Comparison with the arrangement of the elastic tissue in the myxomas favours hypoplasia.

Auricular Thrombus

As many still believe that the tumours described are no more than organized thrombi a brief

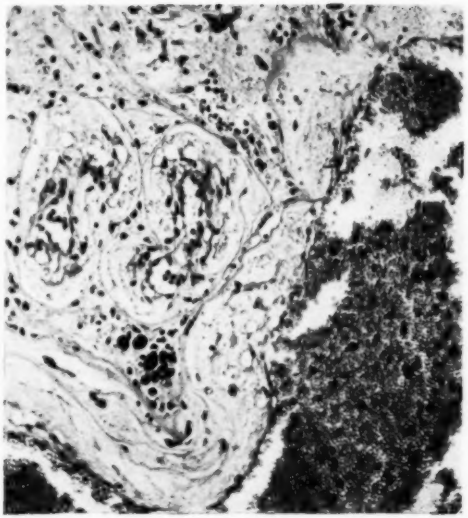


FIG. 7.—Auricular angioma; there are large vascular elements but the smaller vessels in the mucoid matrix are composed of cells very like the lepidic cells of the myxoma. (Haematoxylin and eosin, × 140.)

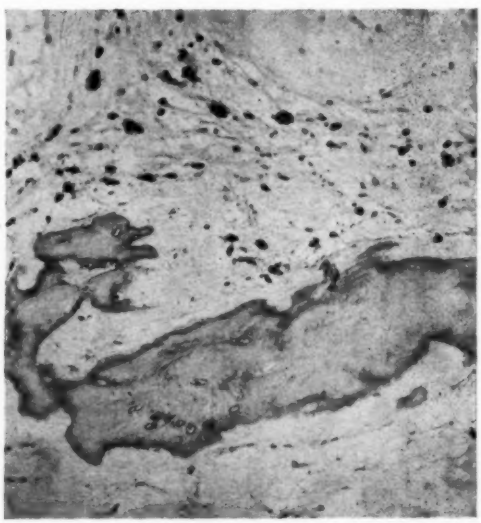


FIG. 8.—Auricular angioma; bone formation in a mucoid matrix containing much free iron. (Perls' method. Neutral red counterstain, × 120.)

account of thrombi is relevant to the discussion. Auricular thrombi are frequently found at necropsy and some show organization of their mural aspect, but a study of 30 such thrombi has failed to demonstrate the slightest resemblance to the angiomyxomas. A pedunculated thrombus in the right auricle does, however, merit description on account of its situation and outward form.

Case Report.—A woman aged 57, known to have suffered for many years from rheumatic heart disease, died from congestive heart failure. At necropsy the heart was greatly enlarged by dilatation and hypertrophy of both auricles and of the right ventricle. There was a moderate degree of mitral stenosis. The right auricle contained an ovoid mass, measuring 2×1×1 cm., attached by a pedicle 0.5 cm. in diameter to the upper border of the fossa ovalis. The mass was firm, reddish ochre, and slightly granular on the surface while the cut surface was ochre, dry, and rather friable with a suggestion of lamination of the outer portion.

Histology.—The greater part of the mass was composed of structureless material devoid of endothelial covering. A zone at the base was invaded by fibroblasts and capillaries and was typical of the granulation tissue of an organizing thrombus (Fig. 9). The only elastic fibres were those associated with formed vessels in and near the pedicle. Metachromatic stromal mucin was present in the amount normally associated with granulation tissue but there was no other mucinous substance. Iron pigment was scanty and confined to histiocytes in the organized area. The elastic lamina of the auricle was intact beneath the thrombus.

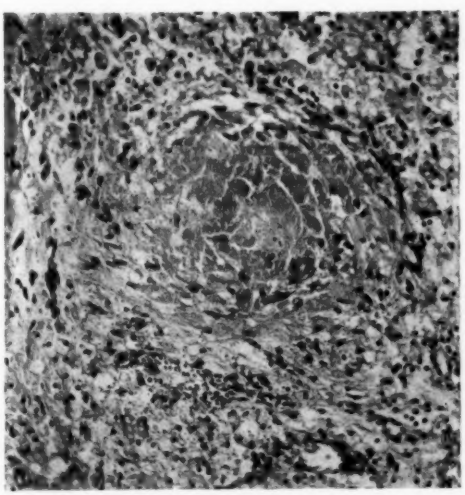


FIG. 9.—The base of an organizing, pedunculated thrombus from the right auricle for comparison with myxoma. (Haematoxylin and eosin, × 150.)

Discussion

Myxomas of the left auricle are clinically silent or may present the symptoms of intermittent mitral stenosis with phases of severe right heart failure and occasionally syncopal attacks due to the tumour sinking into the mitral valve. Auscultatory signs are variable but enlargement of the left auricle and of the right ventricle may be demonstrated radiologically. Von Reis (1949) states that the electrocardiogram is characterized by right ventricular preponderance and expansion of the P waves. Rheumatic and other forms of endocarditis must be excluded.

The four tumours described are all variants of the same intrinsic structure. The first is the best example of the myxoma and the last is an angioma with slight myxomatous attributes. All arise from the same anatomical site, the rim of the fossa ovalis, and all are composed of identical structures namely, vasoformative lepidic cell groups, formed vessels, collagenous and elastic fibres, and myxomatous tissue. The nature of the mucin has not been determined. By Southgate's method or by the P.A.S. technique the three myxomas stain well and diffusely and the angioma stains irregularly and weakly. When stained with toluidine blue all the tumours show areas of metachromatic staining, mainly towards the base in the vicinity of formed vessels. Treatment with hyaluronidase removes the metachromatic staining substance but does not affect the diffuse mucin reaction.

The precise nature of these tumours has been the subject of controversy and, as most workers have seen but one or two, there has been a tendency to unfortunate generalization based on incompletely studied cases. Thorel (1903, 1915) stated that myxomas were always associated with venous thrombosis and that the histological descriptions were those of organization of a thrombus but he relied upon the accounts of others and had apparently no personal experience. Warthin (1916) described mucoid, fibroblastic tissue in organized cardiac thrombi but his observations remain unconfirmed and they bear no resemblance to the histology of myxoma. Husten (1922) agreed with Thorel after studying what seems to have been an organizing mural thrombus in the right auricle. The thrombus theory is an assumption that in special circumstances a thrombus may simulate a neoplasm both macroscopically and microscopically. Against this theory are the facts that auricular myxomas are never laminated, they are histologically unlike an organized thrombus, they do not occur in the auricular appendices, they are rare while thrombi are common, and no transitional stages between thrombus and myxoma have ever

been described. If myxomas were derived from thrombi they should also be found in the ventricles. It is very unlikely that a thrombus several centimetres in diameter would ever organize completely and none of the numerous thrombi, in auricle or ventricle, examined during the preparation of this paper have borne the slightest resemblance to a myxoma. Myxomas, unlike thrombi, are not associated with rheumatic or other inflammatory lesions of the endocardium and the slight thickening of the valve cusps and the chordae tendineae noted in the present series, and previously described by Dexter and Work (1941) in one case, may be attributed to the stress imposed by the mechanically induced deformity of the valve. Ribbert (1924) believed that a neoplasm might arise in myxoid embryonic remnants in the region of the fossa ovalis. Rezek (1938) described an entirely intramural nodule of the right auricle containing epithelial cells and cysts which might well be an early myxoma or at least a complex malformation in which neoplasia might occur. Prichard (1951) has assessed this theory by routine sectioning of the fossa ovalis and although myxoid tissue has not been found he has demonstrated minor endocardial malformations in the form of collections of capillary lacunae lined by swollen endothelial cells. This has been confirmed by personal experience but the malformations so far detected are even smaller than those illustrated by Prichard. However, it is clear that minute foci of hyperplasia do exist in this region.

In the literature the relationship of the tumour and its contained elastic tissue to the elastic lamina of the auricle has either been disregarded or it is merely stated that the elastic lamina of the auricle was intact. All of the angiomyxomas here considered showed a definite elastic lamina close to the surface and in three of them this could be traced to its junction with the auricular elastic fibres, and in Case 1 the hypertrophied auricular elastic tissue split to enclose the tumour. In three the auricular elastic tissue beneath the tumour was diminished. These findings indicate that the tumour originated within the wall of the auricle and, by its expansion, carried with it into the cavity a proportion of the superficial auricular elastic fibres.

The occurrence of neoplasia in malformations is well established and it is suggested that these tumours grow, during adult life, from elements of small pre-existing hamartomas. A comparable process may occur in epidermal moles. They are essentially angiomatous and whether or not they should be called fibromas, myxomas, or endotheliomas is irrelevant if they are regarded as an overgrowth, in varying proportions, of constituents which are normal at some stage in the development of the part. Whether or not they should be considered to be true neoplasms is a matter of personal belief. If anomalous differentiation can be synonymous with neoplasia then the cardiac angiomyxoma is a tumour.

I conclude with what may be the earliest description of one of these tumours, cited by King (1845) from the notes of Thomas Hodgkin. The subject was a woman aged 55.

> "In the left auricle, close to the margin of the foramen ovale, was attached a large polypiform body of about the size of a pullet's egg. The surface by which it was attached was nearly the size of a shilling. Though rather dark and discoloured in some parts, it was generally of a light yellow, and semi-transparent, with some opaque white specks dispersed through it, having some resemblance to the opaque points in soft soap, but rarer and smaller. It was firmer than the ordinary fibrinous concretions which are found in the heart, and was especially so about its root. On fine injection being thrown into the coronary arteries, minute vessels were seen beautifully ramifying through the transparent substance. This polypiform concretion was covered by a thin membrane continuous with that of the lining membrane of the auricle. Though its substance was not disposed in layers, some appearances in it (as, for instance, darkish points of a brownish colour) had a trace of arrangement equi-distant from the surface."

Summary

Four tumours arising in the left auricle near to the fossa ovalis are described. They can be placed in a sequence ranging from almost pure "myxoma" to typical haemangioma. It is considered, upon histological grounds, that they arise from elements of pre-existing intramural hamartomas. As they exhibit slight but definite powers of growth in excess of those of the normal structures they should be termed hamartoblastomas and may for convenience be described as angiomyxomas, although the term myxoma, which indicates a peculiar feature of the collagenous stroma, probably in part degenerative, has little merit.

I am indebted to Mr. G. W. Moore for the photographs.

References

Dexter, R., and Work, J. L. (1941). *Arch. Path., Chicago*, **32**, 995.
Husten, K. (1922). *Beitr. path. Anat.*, **71**, 132.
King, T. W. (1845). *Lancet*, **2**, 428.
Mahaim, I. (1945). *Les Tumeurs et les Polypes du Coeur*. Paris.
Prichard, R. W. (1951). *Arch. Path., Chicago*, **51**, 98.
Reis, G. von (1949). *Acta med. scand.*, **133**, 214.
Rezek, P. (1938). *Virchows Arch. path. Anat.*, **301**, 305.
Ribbert, H. (1924). In Henke, F., and Lubarsch, O. *Handbuch der speziellen pathologischen Anatomie und Histologie*, vol. 2, p. 276. Berlin.
Thorel, C. (1903). *Ergebn. allg. Path. path. Anat.*, **9** (1), 901.
—— (1915). Ibid., **17** (2), 667.
Warthin, A. S. (1916). *J. infect. Dis.*, **19**, 138.
Yater, W. M. (1931). *Arch. intern. Med.*, **48**, 627.

J. clin. Path. (1952), **5**, 345.

POLYETHYLENE GLYCOLS AS EMBEDDING MEDIA IN HISTOCHEMICAL WORK

BY

S. SRIRAMACHARI AND V. RAMALINGASWAMI

From the Nutrition Research Laboratories of the Indian Council of Medical Research, Coonoor, South India

(RECEIVED FOR PUBLICATION MAY 9, 1952)

The embedding and sectioning of tissues in paraffin is a slow process. For histochemical purposes, especially for the demonstration of enzymes, preparation of tissue sections in paraffin is not desirable since the exposures of tissues to high temperatures and to several changes of dehydrating and clearing agents are detrimental to the preservation of enzymes.

Gomori's (1946) experience with the technique of histochemical demonstration of acid phosphatase led him to state that "for some reason, the staining for acid phosphatase turns out patchy, occasionally even negative, when it should be positive. This seems to happen, especially in cases when the pieces have been exposed to the temperature of the paraffin oven for more than an hour or when the temperature of the oven is over 56° C." Several investigators (Danielli, 1946; Stafford and Atkinson, 1948; Rabinovitch, Junqueira, and Fajer, 1949) demonstrated severe loss of acid and alkaline phosphatase activity during paraffin embedding of tissues. Montagna and Noback (1947) thought that acid phosphatase was partially denatured during infiltration in paraffin and felt it advisable to eliminate the paraffin method from the technique wherever possible. Rheingold and Wislocki (1948) also find that in the case of both acid and alkaline phosphatase far more phosphatase is visible in smears of bone marrow than in sectioned material prepared by the paraffin method. In the case of other enzymes also loss of activity is likely to occur due to the several factors discussed above. The paraffin method suffers from the further disadvantage that the sections are not suitable for the demonstration of lipoids. It is clear, therefore, that a rapid method of embedding and sectioning tissues without the disadvantages of the paraffin method is required in histochemical work when enzymes and lipoids are being studied. We present observations on a rapid method of sectioning tissues in polyethylene glycols which is free from these disadvantages.

Synthetic, water-soluble, wax-like polyethylene glycols have been used successfully in recent years for the preparation of tissue sections. They were introduced by Richards, Anderson, and Hance (1942) for preparing sections of muscle of 0.25 μ thickness for electron microscopy. Carsten (1947) dehydrated and embedded eyes in polyethylene glycols and found this technique to be superior to the usual paraffin and celloidin methods. Blank (1949), and Blank and McCarthy (1950) found these compounds advantageous when they prepared sections from a number of tissues for routine staining. Firminger (1950) and Rinehart and Abul-Haj (1951) used them successfully for the demonstration of lipoids.

The polyethylene glycols have the general formula $HOCH_2 (CH_2OCH_2)_x CH_2OH$. Those with an average molecular weight of 1000 and above are wax-like solids.* With increasing molecular weights they become less water-soluble and less hygroscopic. They are inert, non-corrosive compounds and do not extract cell components like fat, glycogen, and nucleoproteins. They are not only good embedding media of varying hardness and melting ranges, but also serve as effective dehydrating agents without causing drastic shrinkage of tissues. Thus, with this method, dehydration, infiltration, and embedding are achieved with the same compound and the entire process is reduced to a few hours. By using the lower members of the polyethylene glycols such as "carbowax 1000," which has a fairly hard consistency and a melting range of 35° C. to 40° C., the temperature of incubation of tissues is also markedly reduced. It was thought, therefore, that these compounds could have an important application in histochemical work, especially for the study of enzymes.

In a critical review of micromethods in the study of enzyme distribution in cells and tissues Holter and Linderström-Lang (1951) state:

"The use of 'carbowax' (polyethylene glycol) as a medium is very promising too, since the

* They are marketed under the trade mark "carbowax" by the Carbide and Carbon Chemical Corporation, New York, and are designated by their molecular weights such as "carbowax 1000," "carbowax 4000," etc.

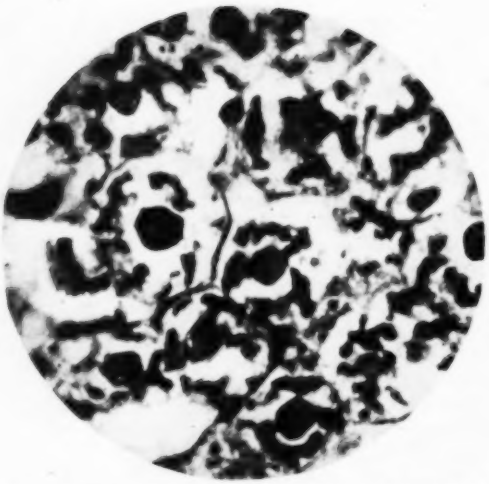


FIG. 1.—"Carbowax" section of rat liver stained with toluidine blue showing basophilic masses in the cytoplasm, × 630.

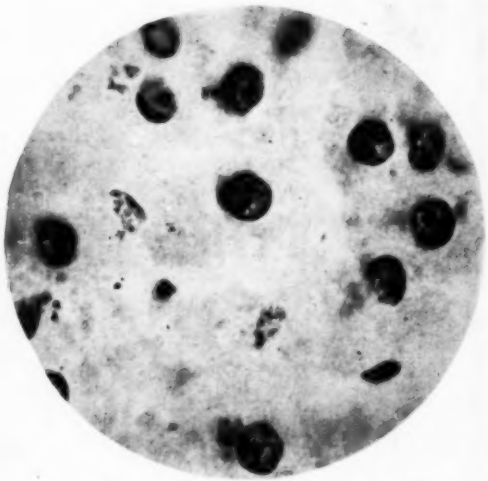


FIG. 2.—"Carbowax" section of the same specimen as in Fig. 1, stained with toluidine blue after treatment of the section with ribonuclease at 37° for one hour. No basophilic material is seen in the cytoplasm, × 630.

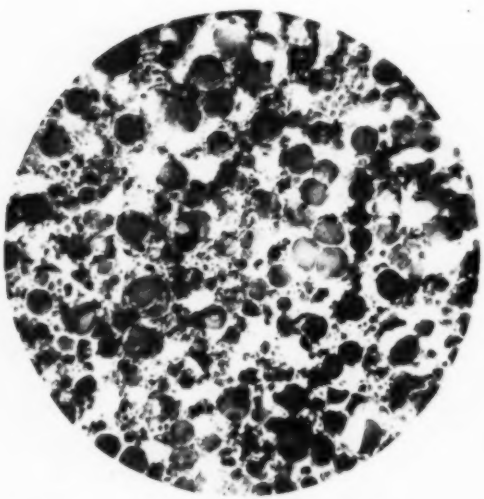


FIG. 3.—"Carbowax" section of liver stained for fat with Sudan IV in propylene glycol from a case of nutritional oedema in an adult. The liver shows severe fatty infiltration and most of the fat droplets are stained, × 280.

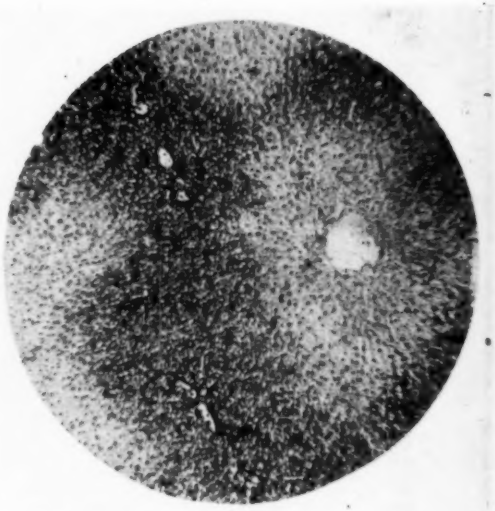


FIG. 4.—"Carbowax" section of liver of a rat injected with carbon tetrachloride 72 hours before. The section has been stained for alkaline phosphatase by Gomori's method. Note the clear-cut zonal distribution of the reaction, centrilobular zones showing no reaction (except in nuclei) and periportal zones showing a marked one, × 70.

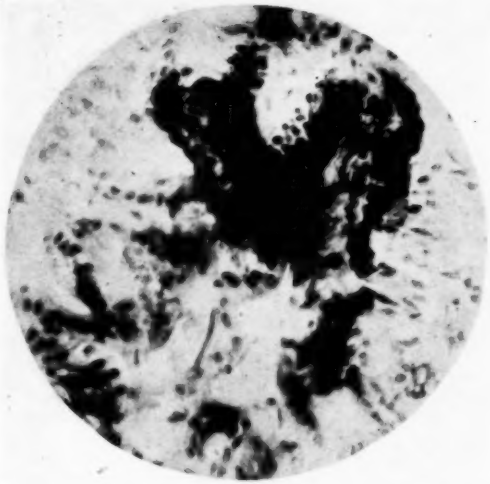


FIG. 5.—"Carbowax" section of rat prostate stained for acid phosphatase according to Gomori. Sites of acid phosphatase activity are seen as dark deposits. The section has been counterstained with safranine, × 280.

FIG. 6.—"Carbowax" section of same specimen as in Fig. 5, stained for acid phosphatase according to Gomori after inactivation of the phosphatase in 0.25% solution of nitric acid. There are no dark deposits. The section has been counterstained with safranine, × 280.

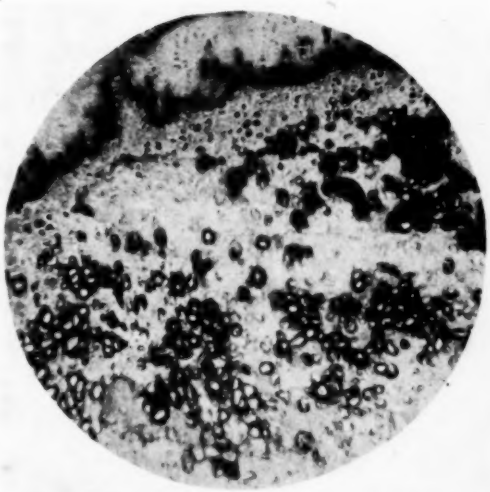


FIG. 7.—"Carbowax" section of tibial cartilage of a rachitic rat stained for alkaline phosphatase according to Gomori. The immature zone is relatively free from the enzyme but the mature zone shows large quantities of it, × 70.

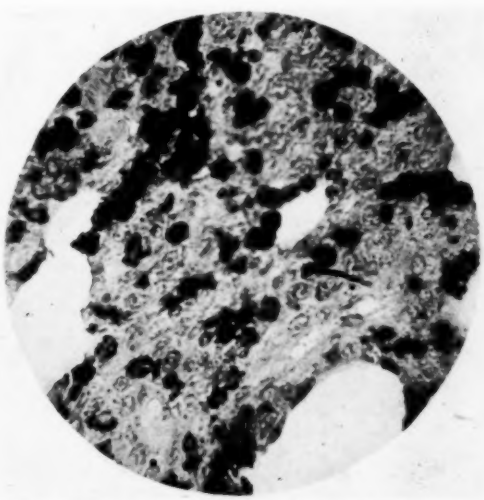


FIG. 8.—"Carbowax" section of guinea-pig lung stained for oxidase by the Winkler-Schultze method showing dark, oxidase-positive leucocytes, × 420.

enzymes do not seem to be extracted from the lyophilised tissue by this water-soluble embedding material."

The distribution of fat, glycogen, nucleoproteins, ribonucleic-acid protein (R.N.A.) and desoxyribonucleic-acid protein (D.N.A.), acid and alkaline phosphatase, oxidase and peroxidase in sections prepared by the more traditional methods have been the subject of study in this laboratory for some time. By employing the polyethylene glycol method we have been able to demonstrate these cell components in a more satisfactory manner.

Material

The material consisted of (*a*) samples of human liver obtained by needle biopsy from persons suffering from nutritional oedema ; (*b*) livers of rats injected with carbon tetrachloride ; (*c*) cartilage and bones of rachitic rats, and (*d*) lungs of guinea-pigs showing severe infiltration with eosinophilic leucocytes. Glycogen, fat, nucleoproteins (R.N.A. and D.N.A.), and alkaline phosphatase were studied in the livers and cartilages. Acid phosphatase was demonstrated in the prostate of normal rats. Oxidase and peroxidase reactions were demonstrated in granular leucocytes in guinea-pig lungs.

Methods

Fixation.—There is no single fixative suitable for the study of all cell components. In the absence of freezing dehydrating apparatus it was necessary to employ different fixatives for the satisfactory demonstration of particular cell components. Thin slices of tissue under 3 mm. thickness were used in all cases. For the demonstration of fat, oxidase, and peroxidase, the tissues were fixed in 10% neutral formalin for 12 to 24 hours. For nucleoprotein (R.N.A. and D.N.A.) 24 hours' fixation in 95% alcohol was employed. For glycogen, fixation in alcoholic formalin (1 part of formalin in 9 parts of absolute alcohol) for 24 hours was employed (Bensley, 1939). For the demonstration of acid and alkaline phosphatases, the methods of fixation recommended by Gomori (1951) were followed. For alkaline phosphatase the tissues were placed in ice-cold 80% alcohol and fixed for 12 to 24 hours in an ice-box. The same procedure was followed for acid phosphatase except that acetone was used in the place of 80% alcohol. It has been shown that enzymatic activity was preserved to the greatest extent by employing these fixatives, although considerable loss of acid phosphatase was still unavoidable (Danielli, 1946 ; Stafford and Atkinson, 1948 ; Rabinovitch, Junqueira, and Fajer, 1949).

Embedding.—After appropriate fixation the tissue is blotted and transferred directly into a bath of "carbowax 1000" which is kept in an incubator at 40° C. "Carbowax 1000" melts completely at this temperature. After one hour the tissue is transferred to another bath of fresh "carbowax 1000," and after another hour is removed from the incubator and embedded in freshly melted "carbowax 1000" using L-shaped metal pieces or paper boxes. The mould is then cooled for about 15 minutes in an ice-box where contact with moisture is avoided by enclosing it in a petri dish. The block, which becomes solid and can be easily separated from the mould by this time, is then trimmed and mounted on a specimen-holder, wooden or metal, in the usual way as with paraffin blocks and is ready for sectioning. The time schedule given above for dehydration, infiltration, and embedding applies to tissues of 2–3 mm. thickness. Thicker specimens need a longer time for infiltration. "Carbowax" blocks should be stored out of contact with atmospheric moisture. They should be wrapped in cellophane paper and preserved in air-tight glass jars, preferably in an ice-box.

The procedure described above is essentially similar to that of Blank (1949). We found that for our climatic conditions during winter (13.7° C.) "carbowax 1000 was superior to a mixture of "carbowax 4000" and "carbowax 1540" as recommended by Blank (1949). A small amount of "carbowax 4000" was added with advantage to "carbowax 1000" to make the blocks harder during summer (18.6° C.). It was also observed that there was no advantage in passing the tissues through increasing concentrations of the "carbowax" in water during the process of dehydration.

Sectioning.—Sections as thin as 1μ have been obtained on a rotary microtome and, as a rule, no difficulty has been experienced in obtaining satisfactory ribbons. The sections are then floated in a dish of water when the wax dissolves quickly leaving the sections floating. They are then mounted on slides coated with egg albumen. The strong surface currents set up during the solution of the wax in water may sometimes tend to disrupt thin, fragile sections. To overcome this difficulty, Firminger (1950) has suggested the use of a 5% solution of "carbowax" in water for floating sections. In our experience with "carbowax 1000" the disruption of sections on floating in water was infrequent. By placing the ribbons directly on wet slides and draining the excess water, disruption of sections could be prevented. All sections are dried thoroughly overnight in an incubator at 37° C. in order to affix the sections firmly to the slides.

Staining Methods.—Glycogen was stained by the periodic-acid leucofuchsin method (Lillie, 1948) and also by Best's carmine method. The results of both the methods were checked by running control sections which were exposed to human saliva for one hour to digest away the glycogen selectively. Fat was stained with Sudan IV in Herxheimer's solution in the usual manner and also with a saturated solution of the dye in propylene glycol according to the method of Chiffelle and Putt (1951). Ribonucleoprotein was stained with toluidine blue and the results were confirmed by pre-treatment of some of the sections with a 0.02% solution of ribonuclease at 37° C. for one

hour followed by toluidine blue staining. Crystalline ribonuclease was prepared from beef pancreas according to the method of Kunitz (1940). Desoxyribonucleoprotein was stained by the Feulgen technique (Feulgen, 1926). Alkaline and acid phosphatase were stained by Gomori's method (1951). To check false positive reactions control slides were always used in which either some essential constituent of the substrate solution was omitted or the enzyme was inactivated. In the case of alkaline phosphatase, glycerophosphate was omitted from the substrate solution, and in the case of acid phosphatase, the enzyme was inactivated by pretreatment of sections with 0.25% nitric acid for five minutes. Sites of oxidase activity were detected by the Winkler–Schultze method and of peroxidase activity by the benzidine peroxidase method (Lillie, (1948).

Results and Discussion

Some of the results are illustrated in Figs. 1 to 8. Glycogen and nucleoproteins stained equally well by this method as by the usual paraffin method. Staining of fat in "carbowax" sections was superior to that in frozen sections as the sections were very much thinner. This made the localization of sudanophilic material in relation to cell components more exact. The method of Chiffelle and Putt (1951), using propylene glycol as the vehicle for Sudan IV, gave better results than the older method which employs Herxheimer's solution. By this method more fat globules were stained and the intensity of staining was improved. Similarly, the staining for acid and alkaline phosphatase was more intense in the carbowax method than in the paraffin method, although the pattern of distribution of their activity was similar in sections obtained by the two methods. This is probably attributable to the shortened period of dehydration and infiltration of the tissues and their exposure to a much lower temperature (40° C.) than with the paraffin method (56° C.).

With the "carbowax" method dehydration, infiltration, and embedding are achieved with the same agent and the entire process is completed within a few hours. This advantage is common to all types of work, whether general or histochemical, but is particularly valuable for histochemical studies. As far as the demonstration of lipoids within cells is concerned, the carbowax technique seems to be already the method of choice (Firminger, 1950; Rinehart and Abul-Haj, 1951). In addition, it is technically useful when handling tiny biopsy specimens of liver for showing fatty infiltration and following its regression on treatment as it is extremely difficult to make frozen sections of these fragments.

For the histochemical demonstration of those enzymes which we have investigated, carbowax embedding is by far the best method. The advantages of this method over the paraffin embedding technique are: (1) The specimens need not be passed through alcohols and the usual clearing agents used for preparation of paraffin sections; (2) the temperature of the infiltrating and embedding baths need not be higher than 40° C.; and (3) the time of incubation in the baths is relatively short. All these factors are important for the preservation of enzymes in the tissues. The loss of enzymatic activity which is known to occur during paraffin embedding of tissues is considerably reduced by this method. However, it must be pointed out that this method does not necessarily ensure accurate localization of enzymatic activity in cells, which continues to be a major drawback in all histochemical work dealing with enzymes.

The method is not free from disadvantages. Chief among them is that it is difficult to obtain ribbons consistent with every type of tissue and that serial sections cannot always be guaranteed.

Summary and Conclusions

A variety of animal and human tissues was dehydrated, infiltrated, and sectioned in solid, wax-like polyethylene glycol compounds.

Glycogen, fat, nucleoproteins, acid phosphatase, alkaline phosphatase, oxidase, and peroxidase were demonstrated.

For some histochemical purpose the polyethylene glycol technique possesses advantages over the earlier methods of preparing the sections.

We are indebted to the National Carbon Company (India) Ltd., representative of the Carbide and Carbon Chemicals Corporation, New York, for their generous gift of "carbowax." Our thanks are due to Dr. H. Lehmann, of St. Bartholomew's Hospital, London, for his interest and helpful criticism. The guinea-pig lungs were kindly supplied by Dr. I. G. K. Menon, Pasteur Institute, Coonoor.

References

Bensley, C. M. (1939). *Stain Technol.*, **14**, 47.
Blank, H. (1949). *J. invest. Derm.*, **12**, 95.
—— and McCarthy, P. L. (1950). *J. Lab. clin. Med.*, **36**, 776.
Carsten, M. E. (1947). *Arch. Path.*, **44**, 96.
Chiffelle, T. L., and Putt, F. A. (1951). *Stain Technol.*, **26**, 51.
Danielli, J. F. (1946). *J. exp. Biol.*, **22**, 110.
Feulgen, R. (1926). Quoted by Gomori, G. (1951).
Firminger, H. I. (1950). *Stain Technol.*, **25**, 121.
Gomori, G. (1946). *Amer. J. clin. Path.*, **16**, 347.
—— (1951). *Meth. med. Res.*, **4**, p. 1.
Holter, H., and Linderström-Lang, K. (1951). *Physiol. Rev.*, **31**, 432.
Kunitz, M. (1940). *J. gen. Physiol.*, **24**, 15.
Lillie, R. D. (1948). *Histopathologic Technic*. Philadelphia and Toronto.
Montagna, W., and Noback, C. R. (1947). *Science*, **106**, 19.
Rabinovitch, M., Junqueira, L. C., and Fajer, A. (1949). *Stain Technol.*, **24**, 147.
Rheingold, J. J., and Wislocki, G. B. (1948). *Blood*, **3**, 641.
Richards, A. G., Anderson, T. F., and Hance, R. T. (1942). *Proc. Soc. exp. Biol.*, **51**, 148.
Rinehart, J. F., and Abul-Haj, S. (1951). *Arch. Path.*, **51**, 666.
Stafford, R. O., and Atkinson, W. B. (1948). *Science*, **107**, 279.

J. clin. Path. (1952), 5, 350.

STANDARDIZATION OF THE DYE TEST FOR TOXOPLASMOSIS

BY

J. K. A. BEVERLEY AND C. P. BEATTIE

From the Bacteriology Department, The University, Sheffield

(RECEIVED FOR PUBLICATION AUGUST 9, 1952)

With the ever-increasing interest being taken in toxoplasmosis more and more requests are reaching laboratories for help in its diagnosis.

Prominent among the diagnostic tests employed is the cytoplasm-modifying-antibody (dye) test introduced by Sabin and Feldman (1948). Briefly, this consists in mixing varying dilutions of the patient's serum with peritoneal exudate rich in *Toxoplasma* from an inoculated mouse, incubating the mixture, and then adding methylene blue. If the patient's serum contains antibody the *Toxoplasma* will not take up the dye. We have been using this test since 1949. It has been our aim to control or correct variable factors as far as possible so that the results obtained from day to day may be consistent within our own laboratory. Our immediate hope in writing this paper is that we may help others to overcome difficulties that we have encountered, and our ultimate hope that from studies such as ours there may evolve a universally accepted technique that will produce consistent results between laboratories.

With these objects in view, we propose to discuss the reagents used in the test and to describe modifications we have thought desirable.

Materials and Methods

Patient's Serum.—When we first started using the Sabin–Feldman test we used freeze-dried specimens in order to preserve the labile, complement-like "accessory factor" present in the patient's own serum. Winsser and Makstenieks's paper (1949) led us to realize that this was unnecessary, as "accessory factor," or "activator" as they call it, can easily be supplied by fresh serum taken from a normal donor. It has not been our practice to inactivate the patient's serum, as we have found no difference between non-inactivated serum and serum inactivated for half an hour at 56° C. Nor have we found the antibody titre of serum to fall during the day or two spent in the post. When received the serum is stored at about 4° C. Dilutions of serum we make up in saline, not in activator serum, for that would result in varying quantities of activator being present in the dilution tubes, and we have evidence that the amount of activator influences the result.

Activator.—The sera of many normal persons contain antibody in small amounts. Such sera are not suitable as sources of activator. When we are selecting a donor we first mix his fresh serum with mouse peritoneal exudate rich in *Toxoplasma*, incubate, and then stain. Only if we find that few, if any, *Toxoplasma* are unstained do we use that person as a donor. Latterly we have developed a pool of suitable donors from members of laboratory staffs and patients with polycythaemia. The separated serum is bottled in 5-ml. amounts and kept at −20° C. until required. Thus stored we find that activator retains its potency unimpaired for at least three months.

Dye.—We use the saturated alcoholic solution of methylene blue described by Sabin and Feldman (1948) freshly made on the day the tests are performed, generally while the tests are in the water-bath. We find that even if only 24 hours old the dye tends to form a dirty precipitate with the *Toxoplasma* suspension-activator-antibody mixture. We have tried six different commercial preparations of methylene blue and find that all give identical results.

***Toxoplasma* Suspensions.**—The strain of *Toxoplasma* we use was obtained from Dr. Cathie, who states (Cathie and Dudgeon, 1949) that it was originally isolated by Sabin in 1939 from a child who had acute encephalitis.

It is maintained by intraperitoneal passage in mice, four mice being on the average inoculated at one time and the interval between passages being from three to five days. No special strain of mice is used, but we find it important, when we are going to use the peritoneal exudate to provide suspension for the test and not merely for passage, to use mice of 8 to 10 weeks old which have been inoculated three days previously. The reason for this is that we find that antibody, which may interfere with the test, develops in the mouse. The sooner the mouse is killed the less of this there will be. Three days is the earliest time at which an exudate sufficiently rich in *Toxoplasma* can be obtained. There is no advantage in using an older exudate for its greater volume, since

the count of stainable *Toxoplasma* is less. Less antibody is produced in young than in older mice (Table I).

TABLE I
RESULTS IN YOUNG AND OLD MICE

Days since Inoculation of Mouse	Average Percentage of Unstained *Toxoplasma* in Peritoneal Exudate		Dye Test on Mouse Serum (Average % Unstained *Toxoplasma**)		No. of Mice Used	
	Young	Old	Young	Old	Young	Old
3	2	10	2	12	4	7
4	21	29	9	25	3	8
5	28	58	13	30	4	10

* 1 in 4 dilution of serum, results corrected to 30,000 *Toxoplasma* mm.^{3} in exudate.

To prepare the *Toxoplasma* suspension we kill as many mice as we consider will be required to provide sufficient exudate for the number of tests we have to do. From one mouse we can obtain 0.5 to 1 ml. and this is enough for four tests. Should the exudate be scanty, we wash out the peritoneal cavity with about 1 ml. of normal saline. The exudate or suspension is heparinized, 2 drops of heparin (100 units/ml. in normal saline) for each mouse exudate, and films are made and stained with polychrome methylene blue. They are examined to make sure that they are rich in *Toxoplasma* and that these are mainly extracellular, for intracellular *Toxoplasma*, not being visibly affected by antibody, are useless in the test.

We make no attempt to standardize the number of *Toxoplasma* in our suspension. It would be difficult to do this and still use the suspension fresh, nor is it necessary, for we find that, using suspensions varying in content between 1,500 and 90,000 *Toxoplasma* mm.^{3}, there is a direct relation between the number of *Toxoplasma* and the serum concentration which will modify 50% of them. We therefore use any suspension with a normal content of *Toxoplasma* and later, when we are reading the test, determine the *Toxoplasma* content and correct our results to a standard of 30,000 *Toxoplasma* mm.^{3}

A point of great importance is that the *Toxoplasma* suspension should be used immediately. *Toxoplasma* in a suspension which has been allowed to stand at room temperature for 40 minutes are twice as susceptible to the action of antibody.

Setting up the Test.—In order that the *Toxoplasma* suspension shall be used fresh one worker prepares it while another sets up the serum dilutions.

The technique of the tests may be summarized as follows:

Tube	1	2	3	4	5	6
Patient's serum	2 drops undiluted	2 drops 1:2	2 drops 1:4	2 drops 1:8	2 drops 1:16	—
Saline ..	—	—	—	—	—	2 drops
Activator	4 drops	4 drops	4 drops	4 drops	4 drops	4 drops
Toxoplasma suspension	2 drops	2 drops	2 drops	2 drops	2 drops	2 drops

The tubes are incubated for one hour in a waterbath at 37° C., and methylene blue added as follows:

Methylene blue ..	6 drops	6 drops	6 drops	6 drops	6 drops	6 drops

The results are read, the final dilutions being as set out below.

Final dilutions	1:4	1:8	1:16	1:32	1:64	Control

With strongly positive sera subsequent tests, including dilutions up to 1:1,024, or higher, are made.

The same pipette is used throughout and is washed with saline between measurements of the different reagents.

The reason for one hour's incubation is that intracellular *Toxoplasma*, if there be any in the suspension, are liberated by disintegration of some of the tissue cells during the first half-hour. A further half-hour must be allowed to permit the antibody to act on them.

After the dye has been added the tubes are put in the refrigerator and each one kept there until we are ready to examine it. We much prefer to make the examination on the day the tests are set up, as the unstained *Toxoplasma* are then much clearer and less shrivelled than if left until the next day.

Reading the Test.—We are now able to take time to determine the number of *Toxoplasma* in our suspension. We make a count in a Neubauer counting chamber of the stained extracellular *Toxoplasma* in the activator control tube. As they take longer to sediment than do red blood corpuscles, time must be allowed for this by leaving the chamber for 10 minutes in the refrigerator.

The percentage of stained and unstained *Toxoplasma* in the various tubes is next determined. To do this we place two drops on a coverslip and place a slide on top. The slide is then turned over and examined under a 1/6 in. objective with the light cut down. Often there are marked variations in the thickness of the film due to irregularities in the slide or coverslip. We try to examine portions of even thickness. Thick portions tend to give an erroneously high count of stained *Toxoplasma*, probably because the unstained are less easily seen and because larger *Toxoplasma* which are less readily modified by antibody are more likely to be found there.

One hundred *Toxoplasma* are identified in each film and the percentage of stained and unstained noted.

We begin with the control. The percentage unstained there is usually less than 20. If it is over 30 we discard the test.

Calculation of Results.—We take as our end-point the highest dilution which will modify (prevent staining of) 50% of the *Toxoplasma* not affected by mouse antibody. Fig. 1 is an example.

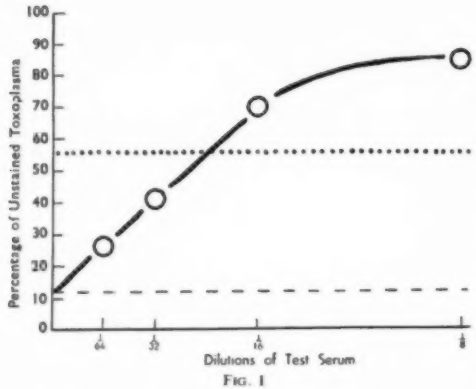


FIG. 1

In the control tube 12% of *Toxoplasma* were found unstained. Half of the remainder is 44%, to which is added 12%. The end titre will be the serum dilution corresponding to 56% of unstained *Toxoplasma*. By interpolation this is found to be a dilution of 1:21.

This was, however, for a suspension containing 3,750 stained *Toxoplasma* in the control tube. Our standard suspension is 30,000 (5,000 in the final mixture). We therefore multiply 1/21 by 5,000 and divide by 3,750, giving a corrected end titre of 1:16.

It should be noted that this is the final dilution of serum after activator and *Toxoplasma* suspension have been added. Sabin and Feldman (1948) record their results in terms of the original dilution of serum. It would therefore be expected that our end titres would be four times as high, but they dilute their peritoneal exudate 1:5. We use ours undiluted. If our contention that there is a direct relationship between the number of *Toxoplasma* and the amount of antibody necessary to modify them is correct, then our results and theirs should be substantially the same. We have some indication that this is so, for we freeze-dried a few specimens of serum and sent them to Dr. Feldman, who very kindly reconstituted and examined them for us (Table II). When we expected them to reach him we reconstituted duplicate freeze-dried samples and examined them. As it turned out our examination was about three weeks before his.

TABLE II

COMPARISON OF RESULTS IN PRESENT SERIES AND IN SERA EXAMINED BY FELDMAN

No.	Present Result	Feldman's Result
206	1:36	1:64 (between 1:64 and 1:256)*
207	1:3·6	1:4 (between 1:4 and 1:16)
208	1:374	1:1,024 (between 1:1,024 and 1:4,096)
210	Negative	Negative
212	1:12	1:4 (between 1:4 and 1:16)
215	1:164	1:64 (between 1:64 and 1:256)
216	1:21	1:16 (between 1:16 and 1:64)

* Words and figures between brackets are ours.

Interpretation

There should be no difficulty in interpretation in active or recently active congenital or acquired infections. Titres in these cases will be in the hundreds and often in the thousands. Where difficulty does arise is in assessing the significance of lower titres in the serum of persons who have choroido-retinitis or some other clinical condition which may be due to infection with *Toxoplasma* several years previously. Some "normal" adults are found to have antibody by this test, although in most cases this is found only in low titres (Table III). It is possible that this may be due to non-specific stimulation. To us it seems more probable that it is specific and due to infection which has not caused clinical manifestations. For a titre to be considered significant it must be shown that it is above that commonly found in persons of the patient's age. We have examined sera from children undergoing tonsillectomy, from patients attending ophthalmic out-patient departments for refraction or removal of foreign bodies, none of whom had choroido-retinis or uveitis, from blood donors, and from members of laboratory staffs who did not work with *Toxoplasma*. From these figures we tentatively deduce that in Great Britain the following titres are likely to be significant.

TABLE III

RESULTS OF TESTS IN "NORMAL" PERSONS

Age	Percentage Reacting to a Titre of					Totals
	Less than 1:4	1:4 to 1:16	1:16 to 1:32	1:32 to 1:64	Over 1:64	
0–9	98	1	0	1*	0	106
10–19	90·5	6·8	2·7	0	0	73
20–9	71	25	2	2	0	101
30–9	75	23	1	1	0	105
40–9	78	19	3	0	0	108
Over 50	71	27	1	1	0	105

* This child was subsequently found to have a squint.

Under 10 years .. .. .. 1:16 or over
10–19 years .. .. 1:32 " "
Over 20 years .. .. .. 1:64 " "

Somewhat similar results have been obtained by Westphal (1950) in Germany. Higher figures are given from Sweden by von Zeipel and Linder (1951).

We realize that titres lower than those we are prepared to accept may be given by the sera of patients who have been infected with *Toxoplasma* in the distant past and are left only with the end-result of that infection. We realize, too, that, as shown by Sabin and Feldman (1949) and Macdonald (1950), babies born of serologically

positive mothers may, without themselves being infected, have passively transferred antibodies. In such cases a further test carried out two months later will show their nature either by a marked fall in titre or by the complete disappearance of antibody.

Fuller knowledge of the significance of the Sabin–Feldman test will emerge from its extended use coupled with careful recording of patients' clinical histories and their physical signs and symptoms. So that the results of different workers may be compared it seems desirable that the test should be standardized. We make no claim that ours is the best method of doing this: it is merely an attempt.

Summary

In order that the results of workers using the Sabin–Feldman cytoplasm modifying (dye) test for the demonstration of antibodies to *Toxoplasma* may be compared it is desirable that a standard technique should be developed. An inquiry has been made into the variable factors present in the test, and attempts have been made to eliminate or correct them. Suggestions are made for a standard technique.

We tentatively suggest that with our method of carrying out the test the following or higher titres should be regarded as possibly significant up to the age of 10, 1 : 16 ; from 10 to 20 years, 1 : 32 ; over 20 years, 1 : 64.

It gives us pleasure to express our thanks to the physicians who sent us sera for examination, to Drs. Sabin and Feldman, who most kindly examined certain sera for us, and to Dr. Cathie, who supplied the strain of *Toxoplasma*.

References

Cathie, I. A. B., and Dudgeon, J. A. (1949). *Journal of Clinical Pathology*, **2**, 259.
Macdonald, A. (1950). *Lancet*, **2**, 560.
Sabin, A. B., and Feldman, H. A. (1948). *Science*, **108**, 660.
—— —— (1949). *Pediatrics*, **4**, 660.
Westphal, A. (1950). *Z. Tropenmed. Parasit.*, **1**, 526.
Winsser, J., and Makstenieks, O. (1949). *Antonie v. Leeuwenhoek*, **15**, 118.
Zeipel, G. von, and Linder, L. A. (1951). *Acta path. microbiol. scand.*, **29**, 229.

J. clin. Path. (1952), 5, 354.

TWO SMALL COLONY VARIANTS OF *STAPH. AUREUS* ISOLATED IN PURE CULTURE FROM CLOSED INFECTED LESIONS AND THEIR CARBON DIOXIDE REQUIREMENTS

BY

J. C. SHERRIS

From the Department of Bacteriology, Radcliffe Infirmary, Oxford

(RECEIVED FOR PUBLICATION MAY 26, 1952)

An occasional small colony variant or "G" form of *Staph. aureus* may often be seen among the normal colonies on first isolating the organism from infected lesions. A pure growth of small colony variants in primary culture is, however, a rarity, and this note concerns two such strains isolated within a week, both of which closely resembled a strain investigated by Hale (1951).

Case Histories

Case 21269/52.—H.N., a machinist, suffered a penetrating wound of the right hand five days before attending the casualty department of the Radcliffe Infirmary. It had become painful and was treated with hot poultices and one intramuscular injection of 500,000 units of penicillin. There was no history of other recent sepsis.

On examination there was an area of cellulitis surrounding the small scar of a puncture wound and the axillary lymph nodes were tender. Radiology revealed no foreign bodies. Twice-daily treatment with 500,000 units of penicillin intramuscularly was instituted and the hand immobilized.

On the following day the lesion was found to be pointing and was incised. Pus and necrotic tissue were removed and a swab of pus sent to the laboratory. Some oedema and discharge continued for three days, but the condition of the hand steadily improved and healing was complete 12 days after operation.

Case 21362/52.—P.H., a student, attended the casualty department suffering from a tender swelling in front of the left ear which was beginning to point. It had been present for 36 hours and was diagnosed as an infected sebacious cyst. The cervical lymph nodes were enlarged and tender. Penicillin treatment of 500,000 units intramuscularly twice daily was begun and magnesium sulphate dressings applied. On the following day the lesion was incised and a deep pouch of pus was found, but there was no evidence of a cyst wall. A swab of pus was sent to the laboratory. Healing was almost complete five days after incision.

This patient had never before been treated with antibiotics.

Laboratory Findings

The findings on primary culture of the swabs from both these patients were identical and are described together.

Two horse blood agar plates were inoculated from each swab, one of which was incubated aerobically and the other anaerobically. A direct film of pus showed, in each case, pus cells and Gram-positive cocci.

After approximately 16 hours' incubation no growth was apparent on the aerobic plates, but numerous small colonies were visible on the anaerobic plates, which, on staining, showed organisms morphologically typical of staphylococci. After 40 hours' incubation a profuse growth of minute colonies was visible on the aerobic plates and the colonies on the anaerobic plate had enlarged and become pigmented to resemble those of a normal *Staph. aureus* grown under similar conditions. Both strains were coagulase positive and as sensitive to penicillin as the *Oxford H. staphylococcus* when tested by the ditch plate method.

Subcultures of the small colonies from the aerobic plates were made on horse blood agar and the plates were incubated aerobically, anaerobically, and in 10% CO_2. One anaerobic culture was made in the presence of soda lime to absorb any CO_2 resulting from the organism's metabolism. These cultures were incubated for 18 hours at 37° C., and the average diameter in millimetres of the isolated colonies is shown in Table I. The colonies which developed in an atmosphere of CO_2 were typical, normal-sized staphylococcal colonies and were both haemolytic and pigmented.

To exclude the possibility that the stimulation of growth was due to the increased humidity in sealed containers, cultures were incubated in an atmosphere of 10% CO_2 in a desiccator. Normal, large staphylococcal colonies developed from both strains under these conditions.

TABLE I

COLONY SIZE (MM.) OF THE STAPHYLOCOCCAL VARIANTS AND OF A NORMAL STAPHYLOCOCCUS GROWN UNDER DIFFERENT ATMOSPHERIC CONDITIONS

Condition of Incubation	Strain		
	H.N.	P.H.	Control
Aerobic	<0·1	<0·1	1·5
10% CO_2	1·0	1·2	1·5
Anaerobic	0·5	0·3	0·5
Anaerobic + soda lime ..	<0·1	<0·1	0·6

The small colony variants were subcultured on to nutrient agar plates and on to blood agar plates at *p*H 6.5, 7.0, and 7.5, and incubated for 18 hours at 37° C. Minute colonies only developed under these conditions, indicating that the inhibition of the staphylococci was not due to the presence of blood nor was their stimulation due to alteration of the surface *p*H of the medium in the presence of 10% CO_2.

Aerobic subcultures of the normal-sized colonies which had developed in CO_2 resulted in a reversion to the small colony type.

On continued incubation of the primary cultures from the original swabs, and also of subcultures of the small colonies, an occasional larger colony developed which bred true on subculture.

The two strains were reported by Dr. R. E. O. Williams to be of different phage-types. Strain H.N. was of phage-type 3*C*+ and strain P.H. was of type 52*A*+.

Discussion

These two small colony variants of *Staph. aureus* appear to be similar to the strain recorded by Hale (1951) in all the properties for which they were tested.

In metabolic studies he showed that the small variant brought about the dismutation of pyruvic acid and the decarboxylation of oxaloacetate less readily than a normal staphylococcus, but that this function was restored after growth in 10% CO_2.

The origin of these strains is of interest. It is possible in both cases reported here that the penicillin injections given before operation may either have selectively inhibited the large colony staphylococci and allowed the development of the small variant, or, alternatively, have altered the metabolism of the parent strain. As the small colony variant was fully sensitive to penicillin, the former alternative seems less probable. In the case reported by Hale (1951) the small colony variant derived from an abscess was also present in the patient's nose, and it is possible that in a small proportion of the population such variants may colonize the nose under natural conditions.

In both the cases recorded here no growth was apparent on the primary aerobic plates after overnight incubation, and it is important to bear in mind the existence of these strains, as their presence might readily be overlooked.

Summary

Two small colony variants of *Staph. aureus* were isolated in pure culture from closed septic lesions.

Subcultures in the presence of 10% CO_2 gave rise to normal-sized staphylococcal colonies.

On primary aerobic culture no growth was visible after overnight incubation, and attention is drawn to the risk of overlooking this variant in routine cultures.

My thanks are due to Mr. J. C. Scott for permission to publish details of his cases, and to Dr. R. L. Vollum for his advice.

REFERENCE

Hale, J. H. (1951). *Brit. J. exp. Path.*, **32**, 307.

J. clin. Path. (1952), **5**, 356.

COLOURING AGENTS FOR USE IN DISC-ANTIBIOTIC SENSITIVITY TESTS

BY

J. H. BOWIE AND J. C. GOULD

From the Departments of Bacteriology, the Royal Infirmary and the University, Edinburgh

(RECEIVED FOR PUBLICATION JULY 4, 1952)

Not only are the number of sensitivity determinations increasing in most laboratories but also the occasions when it is necessary to test pathogens against several antibiotics at the same time. In general, the practical value of sensitivity determinations depends upon the rapidity with which the clinician can be advised as to the most suitable antibiotic for any particular case. Using the disc diffusion technique this work can be expeditiously performed on a quantitative basis without a great deal of additional material or labour (Gould and Bowie, 1952).

The presence of several similar discs on a single plate necessitates some means whereby each antibiotic may be identified. Fairbrother, Martyn, and Parker (1951) have advocated the use of paper discs of a different shape for each antibiotic ; this procedure is impracticable in quantitative sensitivity determinations. The identities of the discs may be written on the back of the petri dish, but the letters may be accidentally erased. Coloured labels may be attached, but this involves additional labour and facilitates confusion. A particular order may be adhered to when placing the discs on the plates, but this method does not eliminate the possibility of error. A satisfactory solution to the problem exists in the use of self-coloured discs. Originally Morley (1945) used blue and white blotting-paper discs to distinguish between sulphonamides and penicillin on agar plates. With the increased number of chemotherapeutic agents available, the problem has become more pressing and there is need of a wide range of distinctive colours.

A brief note without experimental data by Cook (1951) on the use in sensitivity tests of discs cut from Ford's coloured blotterettes encouraged us to communicate with the firm. They presented us with the following dyes in powder form: Ford red ("Clayton" aniline tolamine pink), Ford yellow ("durazol" yellow I.C.I. G.R. 200), Ford blue ("chlorazol" sky blue I.C.I. F.F. 200), Ford orange ("durazol" fast orange I.C.I. R. 150), and Ford scarlet ("durazol" scarlet I.C.I. 4B 150).

After preliminary work with varying concentrations of these dyes in aqueous solution it was found that discs impregnated with dye solutions of 2.5 mg./ml. had a sufficiently distinctive colour ; this persisted over any reasonable period of incubation on any of the common solid media, with the exception of MacConkey's medium, surface seeded with any of the bacteria normally isolated in a routine bacteriological laboratory.

The dyes are fast and consequently have no colouring effect whatever on solid media. For purposes of identification they are unaffected by any constituent of the common solid media incubated aerobically, anaerobically, or with carbon dioxide. From a practical point of view for sensitivity tests, the dyes are not affected by any of the enzymes formed during bacterial growth and they do not affect the adsorptive capacity of the discs. After exhaustive tests with all the organisms likely to be identified in a routine hospital laboratory, it was concluded that they had no inhibitory effect on organisms which would grow on the surface of solid media.

Since the dyes possessed all these negative characteristics essential for our purposes, we carried out further experimental work to determine their effect on penicillin, streptomycin, chloromycetin, terramycin, and sulphonamides.

As a colouring agent for discs in sensitivity tests, it is essential that the dye should have practically no effect on the antibiotic and vice versa. At the start it was found that the dyes were precipitated by streptomycin ; we therefore use uncoloured discs for this antibiotic.

To investigate the stability of a mixture of the dyes and antibiotics, dilutions of each antibiotic were prepared so that the dyed and undyed solutions contained the same initial concentration of antibiotic. The dyes selected for each antibiotic are detailed in Table I.

TABLE I
THE COLOURS USED FOR IDENTIFICATION OF ANTIBIOTICS

Antibiotic	Dye	Colour
Penicillin ..	Ford red	Red
Chloromycetin	3:1 mixture of Ford yellow and Ford blue	Green
Aureomycin ..	Ford yellow	Yellow
Terramycin ..	1:1 mixture of Ford orange and Ford scarlet	Terracotta
Sulphonamide..	Ford blue	Blue

Guided by our preliminary work, the final concentrations of the dyes selected were 0.5, 1, and 2.5 mg./ml. Therefore for each antibiotic there was one undyed solution and three dyed, each containing identical initial concentrations of the antibiotic. At intervals over a period of three months each solution was assayed in quintuplicate. The results of this experiment (Table II) indicate

TABLE II
COMPARISONS IN ACTIVITY BETWEEN DYED AND UNDYED ANTIBIOTIC SOLUTIONS

Initial Concentration of Antibiotic	Antibiotic	No. of Tests	New Assay Values as % of Initial Concentration			
			Within 24 Hrs.	Months		
				1	2	3
100 units/ml.	Penicillin	50	100	95	95	90
	Penicillin coloured	100	100	95	90	90
2,500 µg./ml.	Chloromycetin	50	100	90	85	80
	Chloromycetin coloured	100	100	90	80	75
5,000 µg./ml.	Aureomycin	50	100	90	85	75
	Aureomycin coloured	100	100	90	80	80
1,000 µg./ml.	Terramycin	50	100	60	50	40
	Terramycin coloured	100	100	50	50	50
1,000 µg./ml.	Sulphonamide	50	100	90	85	80
	Sulphonamide coloured	100	100	90	85	80

that dye concentrations between 0.5 and 2.5 mg./ml. do not enhance or diminish the potency of the antibiotics over a period of three months.

The results summarized in Table III represent one experiment designed to investigate the suitability of the dyes for use in disc sensitivity tests. In this instance the organism was *Staphylococcus aureus* and the medium was blood agar. The final concentrations of the dyes in the dye-antibiotic mixtures added to the discs were 0.5, 1, 2.5, and 10.0 mg. per ml. In each case the assays were performed aerobically in statistically significant numbers.

The readings obtained in this experiment indicate that the dyes do not interfere with the

TABLE III
COMPARISON OF COLOURED AND UNCOLOURED DISCS IMPREGNATED WITH ANTIBIOTICS

Discs	No. of Tests	Diameter of Zones of Inhibition (mm.)			
		Within 24 Hrs.	Months		
			1	2	3
Penicillin	100	31	30	30	29
Penicillin coloured ..	100	30·5	30	29·5	29
Chloromycetin	100	28	28	27	26
Chloromycetin coloured	100	28	27	27	26
Aureomycin	100	19	19	19	19
Aureomycin coloured ..	100	19	18	18	18
Terramycin	100	25	24	23	23
Terramycin coloured ..	100	25	23·5	23	22
Sulphonamide	100	25	25	24	23
Sulphonamide coloured	100	25	25	23	23

diffusion of the antibiotic through the medium, that they have no inhibitory or enhancing effect on the antibiotics, and that they do not increase the rapidity with which the potency of the impregnated discs is lost.

Preparation of the Coloured Antibiotic Discs

To colour the discs for quantitative sensitivity tests (Gould and Bowie, 1952) discs are cut from sheets of Whatman's No. 1 filter paper with a hole puncher having a hole-diameter of 6.3 mm.; they are autoclaved or sterilized by dry heat in lots of 100 in 1-oz. universal containers. Solutions of the antibiotics twice the potency per millilitre required for the discs are made by adding sterile distilled water to commercial preparations of the various antibiotics: penicillin 200 units per ml.; chloromycetin 5,000 µg. per ml.; aureomycin 10,000 µg. per ml.; terramycin 2,000 µg. per ml.; sulphathiazole 100 mg. per ml. Streptomycin precipitates the dyes and is therefore used in uncoloured discs, 10 µg. per disc.

Double-strength solutions of the dyes (5 mg./ml. is adequate) are made in the same way and sterilized by steaming for one hour.

Equal parts of the antibiotic and dye solution are mixed and 1 ml. of the mixture added to each bottle of 100 discs; the disc antibiotic content is therefore 1 unit in the penicillin bottles; 25 µg. with chloromycetin, 50 µg. with aureomycin, and 10 µg. with terramycin. The discs may be stored at 4° C., and will retain their potency for several months in the wet state.

The discs are applied to the surface of plates inoculated either for primary isolation or for subculture. The diameter of the zones of inhibition of growth are measured and referred to standard graphs for each particular antibiotic, prepared with a standard organism of known sensitivity.

Other Colouring Agents

With regard to the use of other colouring agents, we investigated several groups of dyes such as representatives of the triphenyl-methane and acri-

dine series, the oxidation-reduction, and the *p*H indicator dyes. As expected on theoretical grounds, these proved to be unsatisfactory for the purpose ; the antibacterial effects of the first and second interfered with our tests, and with all the dyes the colours altered even during normal periods of incubation. Many were not fast.

The use of cellulose or cotton-fast dyes appeared to be the answer and we were able to show that they had no undesirable side-effects.

Summary

The preparation of discs coloured with Ford dyes for use in routine quantitative sensitivity tests is detailed. These dyes provide a full range of stable colours which enable the different antibiotic discs to be easily recognized.

At a concentration of 2.5 mg./ml. the dyes coloured the discs distinctly so that they were recognizable over normal incubation periods on all common media, MacConkey's medium excepted, seeded with any of the bacteria encountered in routine bacteriology. The colours are fast, are not affected by the atmospheres of incubation, and have no effect upon the inhibitory activity of antibiotics. Also, the antibiotics have no effect upon the dyes, with the exception of streptomycin, which precipitates all the dyes and is therefore used with uncoloured discs.

The dyes have no effect on the diffusion of the antibiotic through agar media, and, as far as sensitivity tests are concerned, the coloured discs are as stable as the uncoloured discs.

We wish to thank Professor T. J. Mackie for his help and encouragement. We are grateful to Mr. J. Dick and Mr. K. Marwick for untiring assistance.

We wish to especially thank Mr. D. McLean Wyllie, chief chemist to T. B. Ford Ltd., Snakeley Paper Mill, Loudwater, High Wycombe, who freely gave advice and supplied us with the dyes and their identities.

References

Cook, I. (1951). *J. med. Lab. Tech.*, **9**, 230.
Fairbrother, R. W., Martyn, G., and Parker, L. (1951). *Lancet*, **2**, 516.
Gould, J. C., and Bowie, J. H. (1952). *Edinb. med. J.*, **59**, 178.
Morley, D. C. (1945). *J. Path. Bact.*, **57**, 379.

J. clin. Path. (1952), 5, 359.

TRANSFER OF FLUORESCIN FROM *PS. PYOCYANEA* TO COLONIES OF OTHER BACTERIA

BY

L. HURST AND E. J. L. LOWBURY

From the Medical Research Council Industrial Injuries and Burns Research Unit, Birmingham Accident Hospital

(RECEIVED FOR PUBLICATION JULY 21, 1952)

Fluorescence under ultra-violet irradiation has been described by one of us as a criterion for the detection of *Ps. pyocyanea* on culture plates (Lowbury, 1951). In mixed cultures we have recently found colonies showing typical fluorescence which proved on subculture to be *Bact. coli*. The colonies of the subculture did not fluoresce. It was thought that colonies of these bacteria in the original culture had picked up fluorescin from the medium on which *Ps. pyocyanea* was also growing. The following experiments were done to test this hypothesis.

Experimental

Overnight broth cultures of the strain of *Bact. coli*, which had been found to show fluorescence, and of the strain of *Ps. pyocyanea*, which was growing on the same plate, were streaked across an agar plate at right angles to each other, the streak of *Bact. coli* being interrupted to allow a gap of about 2 mm. between it and the continuous streak of *Ps. pyocyanea*. After the plate had been incubated overnight it was shown that the streak of *Ps. pyocyanea* and the inner ends of the streaks of *Bact. coli* were brilliantly fluorescent under an ultra-violet lamp with a dark glass filter. A subculture of the fluorescent ends of the streaks of *Bact. coli* did not fluoresce.

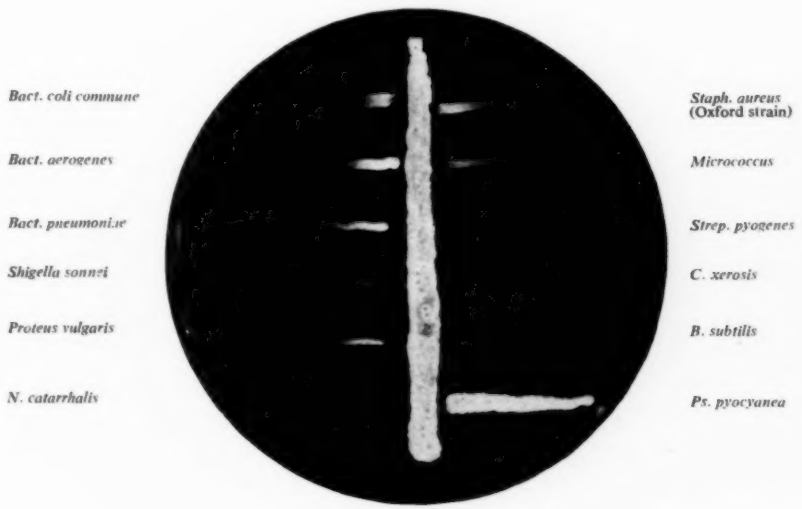


FIG. 1.—Streak cultures of *Ps. pyocyanea* and of other organisms photographed with a Wratten K3 (×2) yellow filter under ultra-violet irradiation. The transfer of fluorescin by diffusion from *Ps. pyocyanea* to some other organisms is shown.

The experiment was repeated with 11 different bacterial species in place of the original strain of *Bact. coli*. A plate of horse-blood agar, containing 4% Davis agar to prevent the swarming of *Proteus vulgaris*, was inoculated in the manner shown in Fig. 1. A loopful of an overnight broth culture of *Ps. pyocyanea* strain S.P. was streaked across the middle of the plate, and streak inocula of broth cultures of the 11 other organisms and of *Ps. pyocyanea* were made with a straight wire, six strains on each side, with gaps of approximately 2 mm. at the inner ends of the streaks. The 11 organisms* used were *Bact. coli commune* 86, *Bact. aerogenes* 8197, *Bact. pneumoniae* 5054, *Shigella sonnei* 8219, *Proteus vulgaris* 4175, *Neisseria catarrhalis* 3622, *Corynebacterium xerosis* 7243, *Bacillus subtilis* 3610, *Ps. pyocyanea* S.P., *Staphylococcus aureus* (Oxford strain), and a strain each of *Streptococcus pyogenes* and of a micrococcus isolated from burns.

After overnight incubation the streaks of *Ps. pyocyanea* and the inner ends of seven of the test organisms showed brilliant green fluorescence under ultra-violet irradiation. Some strains did not show any fluorescence, and this characteristic was not related to the Gram staining reaction; the strongest acquired fluorescence, however, occurred in coliform bacilli. After a further 24 hours' incubation the growth of *Ps. pyocyanea* in some of our tests had ceased to show any fluorescence, though the inner ends of the growth of other organisms were still brilliantly fluorescent; on further incubation this acquired fluorescence was also lost, disappearing first at the inner tips of the streaks. The loss of fluorescence was associated with an increase in green pigmentation.

* We are indebted to the National Collection of Type Cultures for the numbered strains.

Comments

Fluorescin which has diffused from growth of *Ps. pyocyanea* on agar medium is shown in these tests to be absorbed by some organisms growing on the same plate. On continued incubation fluorescence is masked by pyocyanin, first in the growth of the pseudomonas itself, and later by diffusion along the streaks of the other bacterial strains. Plates containing mixed cultures after 48 hours' incubation may show the paradoxical appearance of non-fluorescent colonies of *Ps. pyocyanea* beside fluorescent colonies of *Bact. coli*. Picking of fluorescent colonies to obtain pure cultures of *Ps. pyocyanea* is therefore not a justifiable procedure. However, the presence of green or yellow fluorescence on the growth of a mixed culture still provides a satisfactory index of pseudomonas growing on the medium.

We wish to thank Mr. T. Stone for the photograph.

REFERENCE

Lowbury, E. J. (1951). *Journal of Clinical Pathology*, **4**, 66.

J. clin. Path. (1952), **5**, 361.

MEDIA FOR THE ISOLATION AND DIFFERENTIATION OF PATHOGENIC *ESCH. COLI* (SEROTYPES O 111 AND O 55)

BY

F. RAPPAPORT AND E. HENIG

From the Microbiological Laboratories, the Beilinson Hospital, Petah Tiqua, and the Municipal Hadassa Hospital, Tel-Aviv, Israel

(RECEIVED FOR PUBLICATION MAY 3, 1952)

The connexion between *Escherichia coli* and intestinal disease was observed for the first time by Adam (1923), who described the types of *Bact. coli* found in toxic intestinal diseases of infants. Later he named these bacteria "dyspepsiecoli" (Adam, 1927) and divided them into six groups, A1–A6, according to their fermentative properties in relation to various sugars and alcohols. According to Adam, the two predominant types in clinical cases were A1 and A4, and he designated them *dyspepsiecoli sensu strictori*. Goldschmidt (1933) typed these strains and found that they formed a separate serological group.

Since then many workers (Bray, 1945; Giles and Sangster, 1948; Giles, Sangster, and Smith, 1949; Taylor, Powell, and Wright, 1949; Rogers, Koegler, and Gerrard, 1949; Drimmer-Herrnheiser, and Olitzki, 1951; Dupont, 1951; Braun, 1951) have found in diarrhoea and vomiting (gastro-enteritis) in infants a predominant bacterial flora, *Escherichia coli*, which was named by Bray (1945) *Bact. coli neapolitanum*, by Giles and Sangster (1948) *Bact. coli* type alpha and beta, by Taylor *et al.* (1949) D 433, and by Rogers *et al.* (1949) B.G.T. These strains of *Esch. coli* behave as a distinct serological type. The classification was made by Kauffmann and Dupont (1950), who found that these bacteria belonged to the groups O 111 : B4, O 111 : B4 : H2, O 111 : B4 : H12, and O 55 : B5 : H6.

The growth properties and morphology of these strains are identical with those of other strains of *Esch. coli*, and till now the only means of differentiation has been based on their serological specificity. Even Adam and Aust (1950), who paid particular attention to their biochemical properties, stressed that ". . . as the fermentative properties of these bacteria are unstable and given to fluctuations, it is safer to identify these strains of *B. coli* by means of specific agglutinating sera." A slide agglutination method for identification was also introduced by Bray and Beavan (1948).

We became interested in the possibility of a rapid biochemical differentiation of these pathogenic strains of *Esch. coli*, as it has always been necessary to test serologically all of the colonies diagnosed on McConkey medium as *Esch. coli* in order to be sure that no possible pathogenic colony had been omitted. This procedure, laborious and time-consuming, is impractical for diagnostic purposes.

Method

Some indications of a possible biochemical differentiation were found in previous investigations when Silberstein, Rappaport, and Kolmer (1932) encountered strains of *Esch. coli* that did not ferment sorbitol. Since the serotypes O 111 and O 55 of *Esch. coli* were found to ferment sorbitol slowly or not at all, we tried to include sorbitol in solid media of the type of McConkey. In our final medium the lactose of the McConkey medium was replaced by *d*-sorbitol according to the following formula:

Bacto peptone	17·0 g.
Proteose peptone	3·0 ,,
d-Sorbitol	10·0 ,,
Bacto bile salts No. 3	1·5 ,,
Sodium chloride	5·0 ,,
Bacto agar	13·5 ,,
Neutral red	0·3 ,,*
Crystal violet (0·1% solution)	1·0 ,,
Distilled water	1,000 ml.

This medium was sterilized in an autoclave for 20 minutes at 15 lb./sq. in., poured into petri dishes, and left to solidify. A little faecal suspension in saline was streaked on the surface of the plate which was incubated for 18 to 24 hours at 37° C. Sorbitol-fermenters—that is, most of the non-pathogenic strains of *Esch. coli*—formed red colonies, but patho-

* This high concentration of neutral red (accidentally used in the first assays) was found to inhibit the spreading of *B. proteus*.

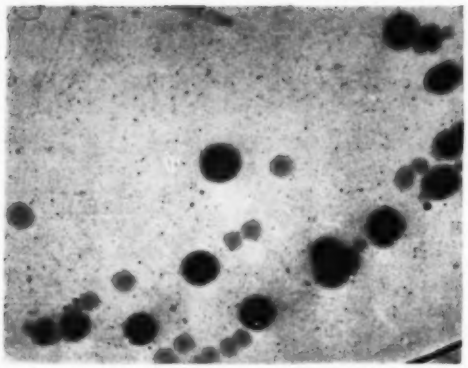


FIG. 1

genic *Esch. coli* grew in colourless colonies similar to those of non-lactose fermenters on McConkey medium (Fig. 1). As some strains of *B. proteus* and some *Shigellae* also grew on sorbitol-agar as colourless colonies, the final differentiation of the pathogenic *Esch. coli* was made on iron-sorbitol-agar slants of the type of Kligler's iron agar, the sugar again being replaced by sorbitol.

Iron-sorbitol-agar slants were prepared as follows:

Beef extract	3·0 g.
Proteose peptone (Difco)	15·0 ,,
Sodium chloride	5·0 ,,
Ferric ammonium citrate (green scales)	0·5 ,,
Bacto agar	20·0 ,,

The mixture was dissolved in 1,000 ml. distilled water and the *p*H adjusted to 7.6 with NaOH. Finally it was distributed in 100-ml. quantities into bottles and sterilized in an autoclave.

A mixture was prepared by grinding in a mortar the following:

Phenol red (powder)	0·15 g.
d-Sorbitol	10·0 ,,
Sodium thiosulphate	2·5 ,,

Of this mixture, 0.25 g. was added to each 100 ml. of melted medium, stirred till dissolved, then distributed into small tubes, autoclaved, and left to solidify in a slanted position.

After 18 to 24 hours of incubation at 37° C. stab inoculation of colourless colonies into this medium gave a slight anaerobic fermentation without producing gas, while normal red colonies of *Esch. coli* grew with the formation of acid and gas. *B. proteus*, which also may form colourless colonies, blackens sorbitol-iron-agar slants due to the production of H_2S.

The colourless colonies of *Esch. coli* gave distinct agglutination with O 111 : B4 and O 55 : B5 sera, while no agglutination was observed in the case of red colonies from sorbitol-agar. As not all colourless colonies are *Esch. coli*, the serological types should be finally established by agglutination with specific sera and by such biochemical reactions as indol- and urease-production and behaviour on Kligler's iron agar and sorbitol-iron-agar. It is possible that new and hitherto undescribed types of *Esch. coli* may ferment sorbitol slowly or not at all. It remains to be seen whether such types, if they arise, are associated with infantile gastro-enteritis. After subcultures on laboratory media some strains of *Esch. coli* types O 111 and O 55 may develop the ability to ferment sorbitol, but this has never been found in the case of *freshly isolated* strains.

Finally it should be mentioned that Kauffmann and Dupont (1950) and Braun (1951) observed that sorbose was not fermented by the pathogenic strains of *Esch. coli*. We are now investigating the effects of the inclusion of sorbose as a differentiating sugar in solid media.

Summary

Strains of *Esch. coli* of the serotype O 111 and O 55 can be biochemically differentiated on sorbitol-agar medium and in sorbitol-iron-agar slants, due to their ability to ferment sorbitol only slowly or not at all. It is suggested that some of the sorbitol-non-fermenting strains of *Esch. coli* may belong to new and as yet undefined serological types.

We wish to thank Mrs. Iza Hirschberg-Glueckman and Mr. Dov Mizrahi for their help in this investigation.

REFERENCES

Adam, A. (1923). *Jb. Kinderheilk.*, **101**, 295.
—— (1927). Ibid., **116**, 8.
—— and Aust, B. (1950). *Mschr. Kinderheilk.*, **98**, 356.
Braun, O. H. (1951). *Z. Hyg. InfektKr.*, **132**, 548.
Bray, J. (1945). *J. Path. Bact.*, **57**, 239.
—— and Beavan, T. E. D. (1948). Ibid., **60**, 395.
Drimmer-Herrnheiser, H., and Olitzki, A. L. (1951). *Acta med. orient. Tel-Aviv*, **10**, 219.
Dupont, A. (1951). *Nord. Med.*, **46**, 1194.
Giles, C., and Sangster, G. (1948). *J. Hyg., Lond.*, **46**, 1.
—— —— and Smith, J. (1949). *Arch. Dis. Childh.*, **24**, 45.
Goldschmidt, R. (1933). *Jb. Kinderheilk.*, **139**, 318.
Kauffmann, F., and Dupont, A. (1950). *Acta path. microbiol. scand.*, **27**, 552.
Rogers, K. B., Koegler, S. J., and Gerrard, J. (1949). *Brit. med. J.*, **2**, 1501.
Silberstein, F., Rappaport, F., and Kolmer, E. (1932). *Zbl. Bakt. I Abt. Orig.*, **124**, 32.
Taylor, J., Powell, B. W., and Wright, J. (1949). *Brit. med. J.*, **2**, 117.

J. clin. Path. (1952), 5, 363.

THE DIFFICULTY CAUSED BY THE FRAGILITY OF THE OVA OF *ASCARIS LUMBRICOIDES*

BY

K. B. ROGERS

From the Children's Hospital, Birmingham

(RECEIVED FOR PUBLICATION APRIL 17, 1952)

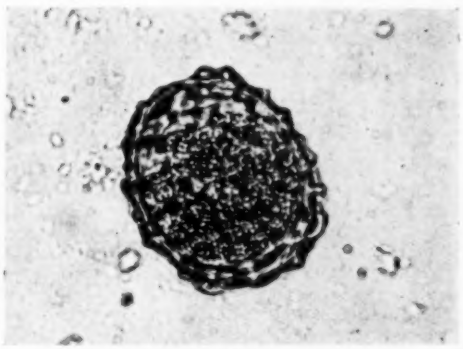
The standard textbooks of clinical pathology and of laboratory technique describe the typical ova of *Ascaris lumbricoides* as being easily recognized, but all refer to the difficulty in recognizing certain of the ova. The following observation may explain why it is sometimes so difficult to recognize them.

When searching for these ova it is convenient to work with relatively thick faecal emulsions and to make a preliminary search with a low power, a 1 in. or ×4 objective, because of their very typical appearance and relatively large size and colour. If an ovum is seen it can be centred and examined with a 2/3 or 1/6 objective to confirm its identity. Todd and Sanford's (1948) description of the typical fertilized ova is that

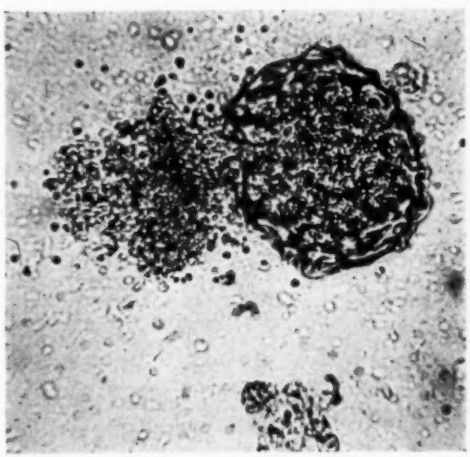
> "they are elliptic, measuring 45 to 60 by 60 to 75 microns, are yellow to brown in colour, and have an unsegmented protoplasm. There is usually a crescentic clear space at each pole, between the contents and the shell. The shell is moderately thick and smooth, and is covered with an irregular albuminous coating."

No mention is made here, or in any other book I have seen, of the ease with which the ova are broken and distorted. All ascaris ova appear to be equally fragile.

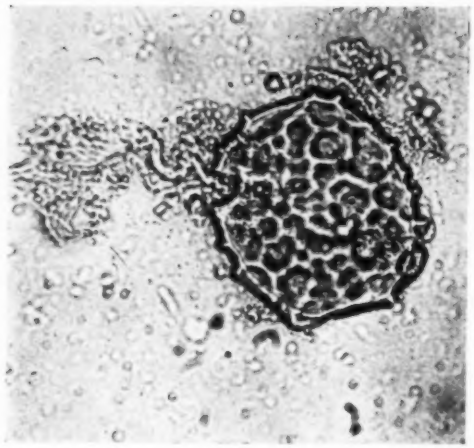
When making a routine examination of a faecal emulsion from a case known to harbour adult ascaris worms no ova could be found in several slides, and a drop of the faecal emulsion was then examined on a slide without a cover slip being placed over it. Several typical ova were then easily found, but what is now recognized to be an automatic, stereotyped, but at that time unconscious movement was observed when a cover slip was placed over this drop. The cover slip did not lie absolutely flat; air bubbles were caught under it and these were gently squeezed out by slight pressure on the cover slip with a pencil point. When this had been done no typical ova were to be seen. A cover slip was then placed gently on another drop of the faecal emulsion. Under the 1 in. objective a typical ovum was moved to the centre of the field, and, while the ovum was observed, light pressure was exerted



A



B



C

with a pencil point. The ovum was seen to burst, the granular contents flowed out, and the general appearance was completely altered when the pressure was released. The photographs illustrate the changes that occur: A is the untouched ova, B after very light pressure, and C after the slightly greater pressure that is usually exerted to make the cover slip lie flat.

This observation may explain why some ascaris ova are difficult to recognize, and it would be best when looking for these ova to make a conscious effort to avoid exerting any pressure on the cover slip.

I should like to thank Mr. J. G. Williamson, the photographer to the Birmingham Children's Hospital, for his co-operation and excellent photographs.

REFERENCE

Todd, J. C., and Sanford, A. H. (1948). *Clinical Diagnosis by Laboratory Methods*, 11th ed. London.

SCHOLARSHIPS IN AID OF SCIENTIFIC RESEARCH

The Council of the British Medical Association is prepared to receive applications for Research Scholarships, as follows:

An Ernest Hart Memorial Scholarship, of the value of £250.

A Walter Dixon Scholarship, of the value of £250.

One or More Research Scholarships, each of the value of £200.

These scholarships are given to candidates whom the Science Committee of the Association recommends as qualified to undertake research in any subject (including State medicine) relating to the causation, prevention, or treatment of disease.

In addition, applications are invited for the award of the following research scholarship:

The Insole Scholarship, of the value of £250, for research into the causes and cure of venereal disease.

Each scholarship is tenable for one year, beginning on October 1, 1953. A scholar may be reappointed for not more than two additional terms. A scholar is not necessarily required to devote the whole of his or her time to the work of research, but may be a member of H.M. Forces or may hold a junior appointment at a university, medical school, or hospital, provided the duties of such appointment will not, in the opinion of the Science Committee, interfere with his or her work as a scholar.

Applications for scholarships must be made not later than March 31, 1953, on the prescribed form, a copy of which will be supplied on application to the Secretary, British Medical Association, B.M.A. House, Tavistock Square, London, W.C.1.

Applicants are required to furnish the names of three referees who are competent to speak as to their capacity for the research contemplated.

J. clin. Path. (1952), **5**, 365.

TECHNICAL METHOD

DEVICE FOR SAMPLING BLOOD BOTTLES

BY

B. A. THOMPSON

From Group 9 Laboratory, Watford

(RECEIVED FOR PUBLICATION MAY 17, 1952)

In constructing the device described below we have used the following criteria: (1) the blood to be cross-matched is the actual blood from the inside of the bottle; (2) the device does not lead to bacterial contamination of the blood in the bottle, in spite of repeated use, thus allowing the contents of the bottle to be cross-matched more than once.

As will be seen from Fig. 1, the device is made mainly from standard parts. It consists of a blood transfusion needle (No. 17, B.W.G.), F, joined to a transfusion adaptor, D, by a piece of rubber tubing 3.75 mm. long, E. Interposed in the centre and interior of the rubber tube is a ¼-in. stainless steel

FIG. 1.—Exploded view of the device.

ball bearing, G. The tubing has an external diameter of 8 mm. and an internal diameter of 4 mm.; the rubber tubing needs to be firm enough to grip the ball bearing and, at the same time, not too rigid to prevent the tubing being pinched by the fingers away from the ball bearing. A dust cap, C, is fitted to the adaptor, consisting of an old record needle mount soldered over at the tip. The device is autoclaved in a plugged test-tube, and is then ready for use (Fig. 2).

To use the device the viscap covering is removed from the top of the blood bottle and the metal cap

FIG. 2.—Device assembled and mounted in sterile tube.

is wiped with alcohol. The needle is then pushed through one of the perforations in the metal cap, and then through the rubber bung up to the shoulder of the needle. The blood bottle is gently inverted, the dust cap, C, removed, the adaptor end, D, flamed, and by pinching the rubber tubing at the site of the ball bearing, thus making a channel between the ball and the inside of the tubing, one or two drops of blood are allowed to run out into a saline tube for cross-matching. The tubing is then released, thereby allowing the tubing to grip the ball bearing again, and the dust cover is replaced before the bottle is righted again. Before the blood is issued for a case the device is withdrawn from the bottle top; or, if the blood is subsequently not required, the device is left *in situ* and the bottle can again be sampled for a further case.

Tests for Sterility

Test 1.—Two bottles containing nutrient broth were fitted with the devices with the dust caps removed. The bottles were inverted, the valves were momentarily opened by pinching the tubing from the ball bearing to moisten the inside of the devices with media; the bottles were then left during the day on the laboratory floor. At night one was incubated at 37° C. and one at room temperature. The contents of the bottle were tested for sterility twice a week for three months, at which time they were still sterile.

Test 2.—The device was interposed between two bottles, one, A, containing glucose indicator broth and the other, B, a culture of *P. vulgaris* in nutrient broth. The dust cap in this case was replaced by a record needle which was passed through the cap of the bottle containing the proteus culture. Before joining the two systems together the valve was momentarily opened by pinching the tubing over the ball bearing and media from A was allowed to flood past the valve; similarly the needle of the culture broth was allowed to fill with infected broth. The two systems were now joined together and set up at an angle to get a slight gravity-feed effect from B to A. This system was incubated at 37° C., and the contents of bottle A were tested weekly for sterility, and the contents of bottle B for viability, the milking process being repeated each time before the two systems were joined. After six weeks the contents of A were still sterile and the proteus organism was still viable.

Test 3.—The device was mounted in a bottle of fresh blood which was kept at 4° C. Thrice weekly for six weeks, by operating the device, cultures of the blood were made under aerobic (air containing 5% CO_2) and under anaerobic conditions; all cultures were sterile.

Summary

A device for removing blood asceptically from transfusion bottles for cross-matching without opening the bottle is described.

J. clin. Path. (1952), **5**, 366.

THE USE OF DEXTRAN SULPHATE AS A BLOOD ANTICOAGULANT IN BIOLOGICAL RESEARCH

BY

M. HALL AND C. R. RICKETTS

From the Medical Research Council Unit, Accident Hospital, Birmingham

(RECEIVED FOR PUBLICATION APRIL 9, 1952)

Several well-known anticoagulants are in use for blood sample tubes, each having some special application, but there is little doubt that the naturally occurring anticoagulant, heparin, would find wider application were it not so difficult to prepare and consequently expensive. Chemically prepared sulphuric esters of polysaccharides are known to have anticoagulant action. One of these, dextran sulphate, has recently been prepared (Ricketts, 1952) in a form suitable for therapeutic use (Walton, 1951). This material, which is readily prepared and therefore cheaper than heparin, may also find application in biological laboratory work.

Some experiments have been conducted to ascertain whether dextran sulphate will give results comparable with the usual anticoagulants in determinations commonly performed in the clinical pathology laboratory. While not comprehensive, these experiments indicate the general suitability of the material for this purpose.

Technique

For the preparation of blood-sample tubes and Pasteur pipettes it has been found convenient in this laboratory to maintain a stock solution of 2% sodium dextran sulphate in aqueous alcohol, prepared by dissolving 2 g. sodium dextran sulphate* in 66 ml. water and adding 33 ml. absolute ethyl alcohol. This solution maintains its potency for several months of storage at room temperature and contains sufficient alcohol to prevent the growth of moulds.

Blood-sample tubes are prepared by moistening the walls of the tube with the dextran sulphate solution, 0.05 ml. of solution per millilitre of blood, and allowing them to dry in an incubator or oven at 37° C. A maximum temperature of 50° C. is recommended for drying. At higher temperatures—for example, 100° C.—the dextran sulphate will char and liberate sulphuric acid. Considerable inconvenience may arise if the anticoagulant in sample tubes is faulty.

Dextran sulphate tubes may be tested for neutrality with an indicator, and for the presence of readily soluble dextran sulphate by adding a little of a weak (5 mg. per 100 ml.) solution of toluidine blue which changes to the metachromatic purple form in the presence of dextran sulphate. The amount of dextran sulphate suggested for sample tubes, 1 mg. per ml. of blood, is considerably more than the minimum required, and corresponds to about 15 international heparin units per ml. of sample.

Many biochemical estimations involve the precipitation of the plasma proteins. When less than 40 mg. of dextran sulphate is present per millilitre of plasma, and precipitation is effected by any of the well-known methods, the dextran sulphate remains adsorbed on the protein precipitate and there is no subsequent interference with chemical determinations. Dextran sulphate does not inhibit urease, so the determination of blood urea is unaffected. No turbidity or difference in reading was observed with Nessler's reagent. Experiments showed that dextran sulphate does not inhibit glycolysis by red cells.

In the field of haematology the requirements of an anticoagulant are somewhat more critical. It was quickly established that there was no effect on the haematocrit determination, but there appears to be a slight though probably negligible effect on the erythrocyte sedimentation rate. Walton (1951) reports that the ordinary haematological determinations, haemoglobin, red cell and white cell counts, are not interfered with.

In the field of physiological research there appears to be scope for an anticoagulant of the heparin type in experiments involving the perfusion of tissue with blood. Since it is not prepared from tissue, dextran sulphate is not contaminated with pharmacologically active substances, e.g., histamine. No doubt other specialized applications will suggest themselves to the research workers concerned. Where a sodium salt is unsuitable it is a comparatively simple matter to exchange the sodium for hydrogen by passage through a column of ion exchange resin. The acid solution of dextran sulphate may then be neutralized with any selected base.

* Dextran sulphate is available from Messrs. Dextran, Ltd., Aycliffe, Darlington.

REFERENCES

Ricketts, C. R. (1952). *Biochem. J.*, **51**, 129.

Walton, K. (1951). *Proc. roy. Soc. Med.*, **44**, 563.

J. clin. Path. (1952), **5**, 367.

ASSOCIATION OF CLINICAL PATHOLOGISTS: 49th GENERAL MEETING

The forty-ninth general meeting of the Association of Clinical Pathologists was held in London from October 2 to 4, 1952.

At the first session the Chairman was Dr. S. G. Platts, and three papers were read.

Benign Polypoid Gastric Tumours

Dr. H. G. H. RICHARDS (Winchester) introduced his subject by describing briefly the clinical features of these tumours, and classifies them into adenomas, pancreatic rests, fibromas, leiomyomas, neurogenic tumours, lipomas, angiomas, and miscellaneous rarities.

The incidence of these tumours was compared with that of peptic ulcers and gastric carcinomas in the Royal Hants County Hospital. The benign group formed about 10% of all gastric tumours (six benign and 53 malignant). The figures were compared with those of other writers.

The morbid anatomy and histology of the individual cases in the various types of tumour were discussed. It was pointed out that epithelial polyps varied from what amounted to simple hypertrophy of the gastric mucosa to frankly adenomatous tumours with early malignant change, all degrees between these extremes being encountered on occasion. A case of polypoid gastric tumour due to a pancreatic rest was described and its typical parenchyma demonstrated; the absence of islet tissue was commented upon.

Next three cases of leiomyoma were described and the similarity of one of them to what in any other situation would be called a neurilemoma was discussed. The central crater in these tumours and its importance to the radiologist was pointed out.

The typical lobulated appearance and ease of diagnosis in a lipoma of the stomach was demonstrated.

The Pathology of the Gastric Arteries, with Special Reference to Fatal Haemorrhage from Peptic Ulcer

Dr. G. R. OSBORN said that although it had been taught for over 100 years that peptic ulcer was related to arteriosclerosis the gastric arteries were practically free from the usual forms of sclerosis. In 1951, 327 partial gastrectomies were performed at the Derbyshire Royal Infirmary. Arteriosclerosis was not seen in any of these.

The gastric vessels were examined in 32 sudden deaths from coronary thrombosis. There were 25 males aged 41–85 (average 61 years), and seven females aged 55–82 (average 74 years). The gastric vessels were all normal with four minor exceptions: three had localized lesions of inflammatory origin, only one (a woman of 74) had an atheromatous plaque, and this was too small to affect the size of the lumen. The splenic and superior mesenteric arteries did not share this immunity from sclerosis. A suggested explanation was that the five series of anastomoses protected the gastric arteries from stresses which produce sclerosis elsewhere.

Arterial lesions related to peptic ulcers should be termed chronic, subacute, or acute erosion. In chronic erosion mucinous infiltration of the intima greatly reduced the size of the lumen; this was the main reason why every patient with peptic ulcer did not bleed to death. In subacute erosion there was an organizing thrombus at the distal (eroded) end; elsewhere the artery was normal. The typical lesion of fatal haemorrhage was acute erosion, which would prove fatal if gastrectomy were not performed in the pre-uraemic stage.

Difficulties in the Use of Antibiotics

Dr. J. D. A. GRAY said that antibiotics had conferred such benefits on mankind that the dangers from their use had tended to be overlooked.

The toxic effects, such as damage to the eighth nerve by streptomycin and the blood dyscrasias after chloramphenicol, were now well known and could usually be avoided. Modern penicillin was non-toxic, but large doses intrathecally were dangerous.

Sensitization of patients to antibiotics had occasionally produced alarming effects. To prevent sensitization a plea was made against the use of penicillin for minor skin ailments.

It was difficult to determine whether the mucous membrane lesions were due to avitaminosis or to a change in the flora.

When penicillin was used as a prophylactic in surgery of the urinary tract or meninges infections with *Pseudomonas pyocyanea* were common and might be prevented by giving a sulphonamide at the same time. Moniliasis might occur after antibiotic therapy in the alimentary, respiratory, and urinary systems, and might be fatal in a case of lung abscess or bronchiectatic cavity.

When an organism became resistant further treatment with the antibiotic might harm the patient and expose his contacts to infection with resistant organisms. The incidence of resistance was increasing; its mechanism was still a subject for speculation; its development could sometimes be avoided and its ill effects overcome by the use of other drugs.

Antagonism between bactericidal and bacteriostatic antibiotics seemed to be limited to cases where the organism was sensitive to the bactericidal drug.

At the second session Professor W. D. Newcomb took the chair.

On the Origin of Visceral Melanomata

Dr. OLIVER C. LLOYD said that primary melanomata occasionally arose in the adrenal or small intestine. He described a case of the former with multiple metastases, in which there was no evidence of a primary tumour in skin, eyes, or meninges; it was the largest in the body. Sixteen other instances had been described, in four of which there were no metastases, so that there could be no doubt that primary melanomata do occur in the adrenal.

Dr. Lloyd then attempted to demonstrate the cell of origin. Specific silver stains for melanin showed granules to be present in 57% of 44 human adrenals examined, irrespective of age or sex. They were found in the cytoplasm of the adrenal medullary cells (phaeochromocytes). The cells affected were often scanty. Fontana's silver solution applied for eight hours or less was not quite specific for melanin, as claimed by Masson, as it also blackens the pigment of ganglion cells. Becker's method, as described by Baker (1938), used 5% silver nitrate at 37° C. for one to two hours. If this were used for only one hour it would blacken nearly all true melanin, including that found in the phaeochromocytes. All adrenal pigments, including melanin, were bleached by nascent chlorine.

He concluded that adrenal melanomata were derived from medullary phaeochromocytes, and that they could be melanogenic was scarcely surprising in view of their common origin from the neural crest with the melanoblasts of the skin and meninges.

REFERENCE

Baker, M. R. (1938). *Arch. Path.*, **26**, 845.

Lingular Biopsy in Mitral Stenosis

Dr. J. B. ENTICKNAP said that specimens had been taken from the lingula segment of the left upper lobe during operations for mitral stenosis in the hope that their examination would be useful in assessing prognosis. They had not been found of much value in individual cases, but some useful information had been derived from the series as a whole.

While the parenchymal changes in the lingula were not typical of the entire lung, the vascular changes did appear to be. Arteriolar sclerosis and hypertrophy were common and both reduced the vascular lumina; as they occurred in similar morbid physiological conditions they had been assessed together by measurement of lumen to wall ratios. Considerable reduction of the lumina was seen, the mean ratio being about 4:1. All the patients had pulmonary hypertension and the lower ratios occurred with the higher pressures; a significant correlation was discovered which could be expressed by the formula:

$$\text{arteriolar lumen/wall ratio} - 21.3 - \frac{\text{mean pulmonary artery pressure}}{3.03}$$

Arteriolar hyalinization was not seen in these sections; a possible explanation was to be found in the fact that the highest pulmonary systolic pressure in these patients (120 mm. Hg) was much less than that found in the brachial artery in cases showing renal arteriolar hyalinization. In two cases with necrotizing arteriolitis the pressures were about 20 mm. lower than the highest in this series and both showed either histological or clinical signs of active rheumatism; it seemed probable, therefore, that arteriolar necrosis in mitral stenosis was related to circumstances other than simple excessive strain. Capillary dilatation was seen in about a third of the cases; their pressures did not differ from those of the remainder and they showed no arteriolar changes.

On October 3 Dr. E. N. Davey took the chair and five papers were read.

The Differentiation of Primitive Leukaemic Cells by *In Vitro* Culture

Dr. D. ROBERTSON SMITH described a method of differentiating myeloblasts, lymphoblasts, and monoblasts by a simple *in vitro* culture.

The bone marrow or peripheral blood is placed in heparinized Tyrode solution and the nucleated cells are separated by centrifugation or sedimentation. Cultures are set up in a medium consisting of equal parts of serum from the patient and Tyrode solution, the final cell concentration being about 2,000 cells per c.mm., and 3-ml. amounts are placed in each of three McCartney bottles. A total and differential count is made and bacteria (either *Staphylococcus* or *Bact. coli*) are added to one tube. The bottles are incubated at 37° C. and examined after four hours and 24 hours.

Myeloblasts mature into promyelocytes and myelocytes after 24 hours, but show no phagocytic activity. Lymphoblasts do not mature or show phagocytic activity *in vitro*, but degenerate rapidly into smear cells. Monoblasts are highly phagocytic in culture and develop into more mature forms. In the Naegeli type of monocytic leukaemia some of the blast cells are phagocytic (monoblasts) and others are non-phagocytic (myeloblasts), whereas in the Schilling type all are phagocytic.

This simple *in vitro* culture method was found to be valuable where routine methods of distinguishing primitive leukaemic cells failed.

The Testing of Plasma and Its Fractions for Anti-haemophilic Potency

Dr. W. R. PITNEY and Dr. J. V. DACIE briefly described a method of studying the generation of thrombin in recalcified citrated plasma by means of subsampling into fibrinogen solution. Normal thrombin generation curves were shown, and contrasted with the far slower evolution of thrombin in cases of haemophilia.

Illustrations were given of the value of thrombin generation curves in assessing the effects of normal plasma or fibrinogen fraction on haemophilic plasma both *in vivo* and *in vitro*, and on the effects of storage at 4° C. on anti-haemophilic globulin.

The Value of Cytochemical Methods in Diagnostic Haematology

Dr. F. G. J. HAYHOE discussed five cytochemical staining methods from the viewpoint of their applicability to routine diagnostic work.

The Feulgen reaction specifically demonstrates desoxyribonucleic acid, which is confined to the cell nucleus. The pattern of nuclear staining resembles that seen in a good Leishman preparation. The intensity of the reaction increases with maturity of the cell. Nucleoli are invariably negative and surrounded by a strongly positive ring of nucleolus-associated chromatin, which makes them readily recognizable. The reaction has a practical application in assessing cell maturity, enumerating nucleoli, and defining nuclear chromatin patterns.

The demonstration of glycogen by the periodic-acid-Schiff reaction gives consistent results and the method is satisfactory and valuable in academic studies. Glycogen is confined to the cell cytoplasm; the erythropoietic series is negative at all stages; the myeloid series shows increasing numbers of positive granules with increasing maturity; eosinophil granules are negative against a positive cytoplasmic background; and lymphocytes and monocytes contain small amounts of glycogen. No significant variations in cell glycogen content seem to occur in blood dyscrasias, however, and the method has no great practical application in diagnostic work.

Sudan black B stains lipids excellently. The myeloid series show increasing numbers of positive granules with increasing cell maturity. Eosinophil granules show a positive periphery with unstained centre. Basophil granules are negative. Lymphocytes are negative, but monocytes contain scattered granules. Although cells from blood disorders do not greatly differ in reaction from comparable normal cells, the reaction is of diagnostic value in demonstrating positive granules in early cells of the myeloid and monocytic series. For this purpose Sudan black seems superior to the commonly used peroxidase reaction.

The techniques of Gomori for acid and alkaline phosphatase demonstration are too unreliable to be of value in routine work.

The Coagulation Defect in Tromexan Therapy

Dr. A. S. DOUGLAS described the investigation designed to elucidate the prolonged one-stage clotting time following the administration of tromexan. Factor V was found to be normal. The addition to tromexan plasma of small proportions of normal serum, which contained no prothrombin, was found to shorten the one-stage clotting time often to normal, while tromexan serum was deficient in this property. This action of serum was described as its factor VII property. Therefore the one-stage test in tromexan therapy reflected a deficiency other than prothrombin. A comparison was made between tromexan plasma and that of a patient with an idiopathic prothrombin deficiency, an excessively rare condition. From a study of this patient's plasma a new two-stage method for the measurement of prothrombin had been devised (Biggs and Douglas, 1952, unpublished data). This patient's plasma, with 10% prothrombin determined by this method, had a one-stage clotting time only slightly prolonged, and his plasma was able to correct the defect in tromexan plasma as well as did normal plasma. By the two-stage technique the prothrombin content of tromexan plasma was estimated and was only occasionally found to be lower than 40% of normal, and usually lay between 40 and 100%. It was concluded that the one-stage clotting time in tromexan therapy was measuring factor VII rather than prothrombin. Using these methods the effects of therapy on prothrombin and factor VII were followed. The factor VII dropped at the onset of therapy to a very low level, the prothrombin some days later, and to a much less extent.

The Significance of Histologically Demonstrable Iron in Sternal Marrow Biopsies

Dr. H. E. HUTCHISON said that the amount of stainable iron in sternal aspirates had been estimated by the Prussian blue reaction. The superiority of histological sections over smears for the purpose of iron staining had been stressed. The results of applying this simple histochemical test routinely to sections prepared from 141 marrow samples were described, and it was shown that, in disorders of the blood, the procedure provided a simple means for the control of iron therapy. It was held as established that in an anaemic subject the absence of stainable iron meant that the patient was deficient in iron, and it was believed that this technical method gave the most decisive haematological indication yet devised for the recognition of iron deficiency. So far as his experience carried him, the converse had also proved true —namely, that if stainable iron were present in the marrow the anaemia would not be improved by the administration of iron whether by mouth or intravenously. Over the past two years, used in this way for the control of iron therapy, the method had not failed, and he had been able to predict when an anaemia would be likely to respond to iron. The method also indicated when enough had been given, and so could be used to obviate the danger of overdosage when iron was being administered by the intravenous route. The method was put forward as a possible means of disintinguishing the hypochromic anaemias of chronic intoxication from those due to iron deficiency.

Dr. A. F. Sladden then took the chair, and four papers were read, ending the morning session.

The Early Detection of Potassium Deficiency

Dr. L. NAFTALIN said that in the post-operative state a correlation existed between the amount of potassium excreted in the urine and the clinical condition. The relation was not a directly causative one in every case, but it had been found that patients in good nutritional status excreted a larger amount of potassium and had an easier clinical course than the

more poorly nourished patients who were unable to excrete potassium. He pointed out that at present this was known only after the event, and he proposed an approach to the earlier detection of potassium deficiency based on the finding of a high serum bicarbonate level, low serum chloride level, normal or near normal serum sodium level, and a history or clinical evidence of reduced food intake. Relevant data from nine case histories were given.

The Interpretation and Principles of Strip Paper Electrophoresis

Professor N. H. MARTIN said that analysis of serum proteins by paper strip electrophoresis had become a routine technique in many laboratories. Because the procedure was still very largely empirical it was vital that its limitations and pitfalls should be appreciated. In the laboratories at St. George's Hospital, London, because of atypical separations liable to be seen when the serum proteins are dried on the filter paper, "wet boundary" techniques have been elaborated. This has involved some loss of clarity of outline. A rigid comparison with the classical electrophoresis has been carried out and it is certain that differentiations achieved by the old technique are not constantly mirrored in the new. The variations in mobilities obtained with purified factions, whether using dextran or albumin markers, indicate that these can only form the crudest means of identification. Quantitative studies show that the binding capacities of the various proteins differ so markedly that correlation between the classical and the paper method by dye elution techniques is impossible, unless some form of correction factor is used. It is impossible, with our present knowledge, to forecast how far empirical correction factors for normal sera are valid for pathological sera. It is difficult to understand the good agreement obtained by some authors between the classical and the paper technique using the Folin reagent if the known difference in tyrosine content of the purest isolated protein fractions is borne in mind. While the Kjeldahl analysis on sectioned strips would be satisfactory, the labour involved seems to negative this as a routine procedure.

With widening experience the hope that the classical and paper strip methods might be equated, a hope which has little justification in theory, is diminishing. It is clear that the technique of paper electrophoresis should, in future, stand alone and, when subject to criticism, must not necessarily invoke apparent qualitative or quantitative similarity with the classical technique as a defence.

The Tubeless Test Meal

Drs. JOHN HARKNESS and JOHN A. DURANT reported their results with Segal's tubeless gastric analysis, which depends on the power of the hydrogen ion of free hydrochloric acid in the gastric juice at a *p*H below 3 to displace the quininium ion of a quininium-resin compound; the liberated quinine is absorbed from the intestine and excreted in the urine, where it can be estimated readily. Segal's quininium-resin indicator is not available commercially in Britain, but an equivalent product has been made from material available here.

(The details of the techniques for the preparation of the quininium-resin, for the estimation of quinine in urine, and for the ward procedure were duplicated for issue to interested persons, and presented separately as a demonstration.)

One hundred and twenty-four duplicate gastric analyses were made in 104 subjects by the ordinary alcohol method and the quinine-resin method. Histamine had to be given to some cases to ensure the secretion of hydrochloric acid for both methods. The patients with free hydrochloric acid excreted considerably more quinine than did the achlorhydric patients; the results of the two groups did not overlap. Four patients showed discrepancies; two patients with free HCl could not excrete quinine because of poor renal function, one achlorhydric patient excreted quinine because of interference by aluminium silicate therapy, and one apparent discrepancy is still being investigated to decide which method is actually yielding the correct results. Patients with achlorhydria due to pernicious anaemia excreted less quinine than those patients with achlorhydria following partial gastrectomy.

Wider trials of the new technique were recommended.

Normal Values for Blood Constituents and Comparative Results from Different Laboratories on the Same Samples

Dr. I. D. P. WOOTTON and Professor E. J. KING said that normal values for most of the commonly determined blood constituents had now been determined. Only some of the constituents are distributed among the population as "normal curves," almost all the remainder showing frequency distributions which are skew and fitted by lognormal curves. For ready reference tables have been calculated showing the limits within which 80% to 98% of the population of healthy people fall.

To determine the possibility of using these tables in other hospitals, agreement between hospitals has been examined. One blood sample and one synthetic solution have been analysed and reported by 25 and 36 laboratories respectively. For most constituents, there are very wide variations in the results, the highest figures reported being two to five times the lowest figures, although results for the solution were better than for the blood. This gives a very bad impression of the reliability of the work of clinical laboratories.

In the case of haemoglobin measurements, an attempt has been made to improve the situation by the Medical Research Council Haemoglobin Standard Scheme, operated from the Postgraduate Medical School of London. Each month a large sample of blood is analysed carefully for its haemoglobin content, using three standard methods of proved accuracy which are described. The blood sample, whose

haemoglobin content is now known with an accuracy better than 2%, is then distributed to interested laboratories in all parts of the world, where it is used to check and calibrate apparatus. At present about 75 laboratories receive the monthly samples.

For the first of the last two sessions on October 4 the chair was taken by Dr. A. Renshaw, when four papers were read.

The Mode of Action of Calciferol in Lupus Vulgaris

Dr. G. WETHERLEY-MEIN briefly discussed the possible ways in which calciferol might act in lupus vulgaris, and the results of an experimental study of this problem were reported.

Standard virulent tubercle bacilli (H37Rv) were cultured and subcultured on a variety of media containing calciferol in concentrations ranging from 50 to 100,000 units per ml. of medium. Certain strains were subcultured at approximate intervals of 30 days for over two years. Comparison with controls showed that calciferol had no effect on the rate of growth, the colonial or bacillary morphology, or the virulence.

Similar studies using lupus strains of attenuated virulence showed that they also were unaffected by culture and subculture in the presence of calciferol.

The sera of various tuberculous and non-tuberculous patients receiving high doses of calciferol (150,000 units daily) had no effect on the growth or morphology of tubercle bacilli.

The rate of healing and histological changes occurring in experimental wounds in guinea-pigs were in no way modified in animals receiving high doses of calciferol.

The findings, coupled with the clinical and experimental observations of Stringer (1948), Jensen (1948), and van der Lugt (1952), suggest that calciferol acts by enhancing the specific cellular reaction of the body to the chemical fractions of the tubercle bacillus following local accumulation of the vitamin in the skin.

REFERENCES

Jensen, T. (1948). *Ann. Derm. Syph., Paris*, **8**, 36.
Stringer, H. W. C. (1948). Ph.D. thesis, Edinburgh University.
Van der Lugt, —. (1952). Personal communication.

The Sensitivity and Resistance of *Myco. Tuberculosis* to Isoniazid

Dr. R. KNOX reported that strains of tubercle bacilli freshly isolated from the sputum of 40 patients with pulmonary tuberculosis were found to be initially sensitive to isoniazid in a concentration of about 0.01 μg. per ml. when a tube-titration method in Dubos liquid medium was used. Standard conditions must be used because the end-point changes with prolonged incubation, partly because of deterioration of the drug, partly because of the development of resistance.

In the course of treatment of a small series of patients with isoniazid resistant cultures developed very rapidly. The numbers of tubercle bacilli in the sputum showed a rapid decrease at first, followed by a reappearance associated with the development of resistance. The relation, however, between resistance in the test-tube and in the patient was not always clear.

Compared with resistance to streptomycin, resistance to isoniazid seems to be produced even more easily, at least in the laboratory, but it does not seem to reach the very high levels which can be obtained by streptomycin-resistant organisms. Isoniazid-resistant organisms retain their sensitivity to streptomycin and vice versa. On the other hand, streptomycin appears to delay the development of isoniazid resistance both in the laboratory and in the patient.

Risk in Routine Tuberculosis Investigations

Dr. W. HOWARD HUGHES reported that, in an attempt to assess the risk to laboratory workers from the spilling of tubercle bacilli during the routine investigation of infected material, cultures were made regularly from the working site after the batch of work had been completed. The method used was to work in large enamel developing dishes lined with cellophane and then to wash this and culture the centrifuged deposit.

Results showed that the spluttering seen when wires were sterilized was of little importance, whereas pouring of homogenized material from the original containers to 15 ml. centrifuge tubes was accompanied by heavy contamination. This spilling could be reduced or eliminated entirely by special care at this stage, and by substituting 25 ml. wide-mouth bottles or 25 ml. centrifuge tubes for the smaller sizes.

Change of staff or alteration of method always caused a temporarily increased risk.

The Renal Localization of *Ps. Pyocyaneus* in Mice

Dr. R. H. GORRILL said that the pathogenicity of *Ps. pyocyaneus* was tested by injecting varying numbers of organisms intravenously into groups of white mice. It was found that with large numbers of organisms the mice died of septicaemia in one or two days. When the dose was lowered the mice survived the first two days and then died within the next fortnight of renal carbuncles. In all 12 strains were tested, derived in equal numbers from wound and urinary tract infections, and the same results were obtained with them all.

The earliest renal lesions were seen at 20 to 26 hours following injection of a suitable dose of *Ps. pyocyaneus*, and consisted of a focal embolic nephritis.

When the distribution of bacteria was followed in the blood, lungs, liver, spleen, and kidney following intravenous injection, it was found that the blood became sterile in 12 to 18 hours and in the lungs in 24 to 48 hours. The liver showed progressively fewer organisms throughout the experiment, but rarely became sterile. The spleen remained about constant or fell, but the kidneys showed a progressive rise in bacterial count.

It is suggested that owing to its special anatomical and physiological properties the kidney tends to filter

out all types of particulate matter passing through it. When pathogenic bacteria like *Ps. pyocyaneus* are filtered out they multiply and rapidly attack the kidney tissue producing renal abscesses.

At the last session Dr. A. G. Shera took the chair.

Physiological Variations in the Blood Count

Dr. JOHN MARKS said that the value of a laboratory test depended on the accuracy of the method balanced against the range of physiological variation.

The magnitude of this physiological variation and the factors involved were discussed. Venous blood gave lower results than capillary and was used throughout.

Age.—Variations in age have no effect on the blood count.

Sex.—The red-cell counts are well known to be lower and the sedimentation rate higher in the female. The leucocyte count is also significantly higher in females.

Time of Day.—The blood count in both red and white cells is lowest in the early morning and rises to a maximum in the afternoon.

Exercise.—Even moderate exercise produces a rise in the leucocyte count.

Environmental Temperature.—During acclimatization to heat there is a fall in the red-cell count. Exposure to cold causes a rise.

Menstruation.—No significant differences occur during the various stages of the menstrual cycle.

It was suggested that serial counts should be performed under "basal" conditions—that is, in the early morning, with activity and temperature change restricted. A set of basal normals was given. This range was smaller than the "random" range for routine diagnostic counts on capillary or venous blood with the causes of variation not controlled.

The values quoted differ from the accepted range.

Papain-treated Red Cells in the Detection of Rhesus Antibody

Dr. K. GOLDSMITH said that papain was a proteolytic enzyme extracted from a plant called carica papaya, or pawpaw. Like trypsin, it might be used to render a saline suspension of Rhesus-positive cells agglutinable by incomplete Rhesus antibody.

The method used was modified from a technique described by Kuhns and Bailey (1950). Six hundred and fifty maternal sera were examined and only two false positive results were obtained, these both being due to cold agglutinins.

Twenty-one sera containing Rhesus antibody were examined, and in no case was agglutination absent when papain-treated cells were used. The antibody titre was noted, using saline, albumin, antiglobulin, trypsin, and papain techniques. In all cases the titre with the papain method was as high as, if not higher than, with the others.

During tests with papain-treated red cells in other blood-grouping systems enhancement of agglutination was noted with anti-A, anti-B, anti-P, anti-M, anti-N, anti-Le^a, and anti-Le^b. No agglutination occurred with anti-Fy^a.

Papain is a readily obtainable laboratory reagent, cheaper than commercial bovine albumin or crystalline trypsin. Rouleaux formation is largely obviated, and the technique seems to be as sensitive for routine use as any other yet available.

REFERENCE

Kuhns, W. J., and Bailey, A. (1950). *Amer. J. clin. Path.*, **20**, 1067.

The Conservative Treatment of Haemolytic Disease of the Newborn

Dr. J. F. HORLEY reported that a series of cases of haemolytic disease of the newborn was analysed. It was collected during four years at Plymouth, and artificially produced prematurity was not featured. Eighty-three cases of maternal Rh sensitization resulted in 11 stillbirths, 11 deaths (seven from kernicterus, one moribund at birth, one from alimentary haemorrhage, one from heart failure, one from pneumonia), 52 living, and nine Rh-negative babies. The living babies were followed up, and only one, a case of neonatal kernicterus with permanent nervous sequelae, was abnormal.

The treatment used was conservative, the babies being transfused only when anaemia demanded it. By this method the cases divided into two syndromes: one (82% in this series) a haemolytic anaemia proportional in severity to the maternal antibody titre adequately and simply treated along the lines indicated; the other kernicterus. It was suggested that it was unsound to treat by exchange transfusion a series of cases in order to avoid the occurrence of kernicterus, and that as an alternative the babies should be allowed to declare themselves into one of the two groups and then treated accordingly, the haemolytic anaemia group by simple transfusion and the kernicteric group by exchange transfusion in the pre-kernicteric stage. In the absence of more specific tests jaundice out of proportion to the haemolytic process, as shown by the rate of fall of haemoglobin, could be used as an indication for exchange transfusion. By this means babies with an excellent prognosis would not be endangered in trying to save those of more uncertain outlook.

The foundation lecture was given by Dr. S. C. Dyke (Wolverhampton), with the retiring President, Sir Lionel Whitby, C.V.O., *M.C.*, in the chair, on **The Development of the Concept of Pernicious Anaemia: Landmarks and Personalities.**

Professor R. J. V. Pulvertaft delivered the presidential address on **The Individual and the Group in Modern Medicine.**

During the meeting a programme of films and demonstrations was given.

J. clin. Path. (1952), **5**, 373.

ABSTRACTS

This section of the JOURNAL *is published in collaboration with the abstracting journal,* ABSTRACTS OF WORLD MEDICINE, *published by the British Medical Association. In this* JOURNAL *some of the more important articles on subjects of interest to clinical pathologists are selected for abstract, and these are classified into four sections: bacteriology; biochemistry; haematology; and morbid anatomy and histology.*

BACTERIOLOGY

Treatment of Chronic Shigella Infection in Children with Oral Polymyxins. LIEBERMAN, D., AND JAWETZ, E. (1951). *Pediatrics*, **8**, 249.

The authors treated with oral polymyxins 23 mentally defective children who were either asymptomatic carriers of or chronically infected with *Shigella flexneri*. Organisms were isolated throughout the investigation. Polymyxin B or E was given in 3 divided doses daily (total 15 to 20 mg. per kg.) for 10 days. All stool cultures were positive at the outset and negative at the end of polymyxin treatment; during the subsequent 8 weeks of isolation stools became positive in only 3 of 23 cases. These results compare favourably with those obtained in 49 similar cases treated with sulphonamides or other antibiotics. No toxic side-effects of polymyxin were observed, suggesting that no significant gastro-intestinal absorption took place.

The authors consider that this drug might be used to treat the entire population of institutions with a high endemic incidence of bacillary dysentery. *Peter Story.*

Some Observations on Survival of Pathogenic Bacteria on Cotton-wool Swabs. Development of a New Type of Swab. RUBBO, S. D., and BENJAMIN, M. (1951). *Brit. med. J.*, **1**, 983.

Different species of bacteria survive for greatly varying periods of time on cotton-wool swabs (as sent to laboratories for examination). The amount of moisture in the swab and the batch of cotton-wool used influence the survival times, but not uniformly for all bacterial species. A new type of swab was devised and found valuable for routine use, as it favoured the survival of bacteria, with the possible exception of *Neisseria*. Wool was rolled on wooden applicators and dipped in ox serum. After drying in an incubator the swabs were sterilized in the autoclave. This treatment made the cotton-wool adhere firmly to the swab stick.

Scott Thomson.

A New Method of Demonstrating Vincent's Spirochaetes. VAGO, S. C. (1951). *Schweiz. Z. allg. Path.*, **14**, 34.

The material is spread thinly on a slide and dried in the air. Neither heat nor fixation is necessary. Stain with a saturated aqueous solution of the disodium salt of dibromo-hydroxymercuri-fluorescein (mercurochrome) for 5 minutes. After the mercurochrome, stain for 5 minutes with a saturated aqueous solution of pyoktanin (Merck's methyl violet). Wash in water and dry. Both stain solutions keep indefinitely and can be made with either ordinary or distilled water. At least 3 minutes in the stains should be given, but after that time the exact duration does not matter. Differentiation is not possible and the *p*H of the solutions used makes no difference, so that the method is simpler than the methods of Gram and Giemsa.

The spirochaetes are stained an intense bluish-black on an unstained background, but the fusiform bacilli often present with them, and also cells and other micro-organisms, take the stain with the same intensity. Details in the organisms are not made visible, but the outlines of the spirochaetes are well preserved. The spirochaetes appear about one-fourth as big again as those stained by silver-impregnation methods, about half as big again as those stained by the methods of Griesbach or Tunnicliff, and almost twice as big as those stained by the methods of Gram or Giemsa. *G. Lapage.*

BIOCHEMISTRY

Renal Function Studies of Severely Burned Patients. A Preliminary Report. HAYNES, B. W., DEBAKEY, M. E., and DENMAN, F. R. (1951). *Ann. Surg.*, **134**, 617.

The results of 16 renal function studies on 8 severely burned patients are reported. All the patients were given adequate treatment, including administration of fluid, and only one of them died. An unusual pattern of renal function was observed, characterized by an increased glomerular filtration rate, a normal renal plasma flow, and an increased filtration fraction. The return to normal function took as long as 2 to 2½ weeks. During the same period the maximum tubular excretory capacity remained within normal limits. The mechanism of these alterations is unknown. The authors, however, discuss some physiological factors that may work in the same direction, such as overhydration, increased thyroxin output, hyperadrenalinism, pyrogenic response, hypoproteinaemia, and increased adrenaline activity. They point out that similar functional changes have been observed after major surgical operations in the immediate post-operative period. *A. Swan.*

Gout. Observations on the Effects of Drugs on Plasma Uric Acid and Urinary Uric Acid. KERSLEY, G. D., MANDEL, L., and BENE, E. (1951). *Ann. rheum. Dis.*, **10**, 353.

The authors kept 13 patients suffering from classical gout under observation in hospital. Daily estimations

of plasma uric acid and urinary uric acid were made before, during, and after administration of drugs which were known or believed to influence uric acid levels in blood and urine. The drugs administered were colchicine, sodium salicylate, glycine, "carinamide," cinchophen, and "probenecid." Carinamide (phenylmethane sulphonanilide) was used because it might reduce tubular resorption of uric acid by the kidneys. These drugs were given over 3-day periods.

It was found that while colchicine was clinically effective it only reduced the plasma uric acid in 3 of 12 patients and increased the urinary uric acid in 2. Sodium salicylate reduced the plasma uric acid in 8 of 12 patients; glycine caused a fall in plasma uric acid in 6 and an increase in urinary uric acid in 5. Six patients received 0.5 g. of carinamide 4-hourly. None of them showed a decrease in plasma uric acid, but in 2 the urinary uric acid rose; when the dose to 3 patients was quadrupled all showed a fall in plasma uric acid and 2 a rise in urinary uric acid. Cinchophen caused a fall in plasma uric acid in 10 of 11 patients and a rise in urinary uric acid in 6. Probenecid caused a fall in plasma uric acid and a rise in urinary uric acid in all of 8 patients.

W. Tegner.

HAEMATOLOGY

Epidemic of Homologous Serum Hepatitis Apparently Caused by Human Thrombin. LESSES, M. F., and HAMOLSKY, M. W. (1951). *J. Amer. med. Ass.*, **147**, 727.

Two freeze-dried, Seitz-filtered preparations of thrombin derived from pooled human plasma have been found to be associated with the agent of homologous serum hepatitis. In the epidemic recorded in this paper all the 221 cases arose post-operatively over a period of 10 months in patients in one hospital where absorbable gelatin sponges soaked in human thrombin had been used. The epidemic followed a change from bovine to human thrombin. The incubation period in these cases varied from 66 to 137 days, with a mean of 103 days. One patient died as a result of the infection.

Similarly contemporary epidemics of hepatitis were observed in two nearby hospitals which had employed human thrombin preparations for surgical purposes.

G. Payling Wright.

Cryoglobulinaemia in Multiple Myelomatosis. BLADES, A. N. (1951). *Brit. med. J.*, **1**, 169.

References have been traced to about a dozen cases in the literature showing cryoglobulinaemia, a term which implies the presence in the plasma of a globulin which precipitates or solidifies on cooling to about 30° C. The phenomenon is reversible on warming to 37° C. and the changes may be repeated indefinitely. Such globulins occur in multiple myelomatosis and have also been found in endocarditis lenta, kala-azar, nephrosis, and in infective arthritis. In the case reported here the diagnosis of multiple myelomatosis was suggested by the finding of cryoglobulinaemia. The author reviews the hitherto published observations on the serum containing this abnormal globulin, and also discusses the nature of this abnormal protein occurring in multiple myelomatosis. *S. Karani.*

Eosinophil Counting: a Modification of Pilot's Method. MACFARLANE, J. C. W., and CECIL, G. W. (1951). *Brit. med J.*, **2**, 1187.

Attention is drawn to Pilot's fluid for the enumeration of eosinophils. Counting is much easier and quicker than with Randolph's fluid, while the disadvantage of Rud's fluid is also overcome. A source of error in the use of the fluid is the clumping of leucocytes on standing. This may be eliminated by the addition of heparin to the fluid in the concentration of one unit per millilitre. There is some evidence that this clumping is due to the formation of fibrin within the pipette.—[Authors' summary.]

MORBID ANATOMY AND HISTOLOGY

Cancer as a Sequel to War Wounds. GILLIS, L., and LEE, S. (1951). *J. Bone Jt Surg.*, **33-B**, 167.

Twenty-four cases of war wounds in which squamous carcinoma developed are described. No changes occurred less than 18 years after and are still occurring 30 years after the original injury; at least 13 of the patients have died of cancer. There are 16 examples of sinus cancer, all occurring in the lower limbs or buttocks. There is a history of a long-standing sinus, with bone infection for many years, and deep in the sinus the tumour occurs; some of these tumours are highly malignant. There are 7 examples of scar cancer, all arising in thin, poorly nourished, unhealthy scars.

It is emphasized that patients with long-standing sinuses should be kept under close observation, and should carcinomatous changes supervene surgery must be radical. An unhealthy scar should be excised and, if necessary, replaced by a pedicle flap. *Peter Ring.*

Neoplastic Cells in Bone Marrow Aspiration. [In English.] RUBINSTEIN, M. A., and SMELIN, A. (1951). *Acta haemat., Basel*, **5**, 292.

The recognition of cells from metastases of malignant neoplasms in bone-marrow smears is not always easy, but in a series of 100 cases of advanced malignant disease 45 showed "malignant cells," mostly in clumps, in smears of sternal or iliac bone marrow. In 21 of 35 cases of mammary carcinoma, in 3 of 14 cases of gastro-intestinal carcinoma, 4 of 10 cases of lung cancer, and [surprisingly] in 11 of 21 cases of carcinoma of the genito-urinary tract (which included the prostate, ovaries, and ureter) neoplastic cells were found. Another feature of this investigation was the hypoplasia or aplasia observed in the bone marrow in about 75% of cases. When the marrow showed normal or increased cellularity neoplastic cells were not found so frequently as in aphasia. Anaemia and abnormalities in the peripheral blood were seen in only 10% of cases with neoplastic cells in the marrow. Sometimes bone-marrow biopsy showed neoplastic cells even though there was no radiological evidence of metastases. *E. Neumark.*

INDEX TO VOLUME V

A

Age, influence upon serum iron in normal subjects, 10
Agglutination reversal, mechanism of, 332
Amino-aciduria and copper metabolism in hepatolenticular degeneration, 16
Amperometric titration, determination of blood glutathione by, 30
Amyloidosis, primary, with renal and myocardial failure, 54
Anaemia, Cooley's, diagnosis of carriers of, 154
— iron storage and, 281
— megaloblastic, of pregnancy, folic acid therapy in, 329
— of pregnancy, the bone marrow in, 145
Anaemias, megaloblastic, vitamin B_{12} concentrations of serum and urine in, 129
Angiomyxoma of the auricle, histogenesis of, 339
Anisotropic non-silicious crystalline inclusions in a dysgerminoma, 239
Antibiotic resistance in Gram-negative bacilli, 169
— sensitivity of pathogenic staphylococci, 165
— — tests, disc-, 356
Anticoagulant, dextran sulphate as, 366
Arteriopathy, necrotizing pulmonary, associated with pulmonary hypertension, 36
Arteriosclerosis, coronary, and calcification in infancy, 175
Ascaris lumbricoides, fragility of ova of, 363
Aseptic filling of screw-capped containers, 290
Aspiration and sternal biopsy needle, combined, 112

ASSOCIATION OF CLINICAL PATHOLOGISTS:
48th General Meeting, 213
49th General Meeting, 367

ASTALDI, G., and TOLENTINO, P.: Studies on the pathogenesis of thalassaemia, 140
Auricle, histogenesis of angiomyxoma of, 339

B

Bacilli, Gram-negative, antibiotic resistance in, 169
BEATTIE, C. P., *see* Beverley, J. K. A., and Beattie, C. P.
BEVERLEY, J. K. A., and BEATTIE, C. P.: Standardization of the dye test for toxoplasmosis, 350
BIDMEAD, D. S., and WATSON, D.: The determination of blood glutathione by amperometric titration, 30
Blood, anticoagulant, dextran sulphate as, 366
— bottles, device for sampling, 365
— film for differential counts, new type of, 108
— glutathione determination by amperometric titration, 30
— — — by nitroprusside method, 25
— in urine, orthotolidine hydrochloride test for, 212
— salicylate estimations, micro-method for, 208
— serum protein-bound carbohydrate after injury, 194
— tissue uric acid values, 317
— transfusion, comparative study of direct matching techniques in, 102
Bone-marrow embolism, 63
— — human, culture *in vitro*, 67
— — in anaemia of pregnancy, 145
— — in Paget's disease, 86
— — value of rib biopsy in study of, 246

BOOK REVIEWS:
BLANC, W. A., *Syndromes Nouveaux de Pathologie Adipeuse*, 1951, 118
CAMERON, G. R., *Pathology of the Cell*, 1952, 296
DE MUYLDER, C. G., *The "Neurility" of the Kidney*, 1952, 297
DYKE, S. C., ed., *Recent Advances in Clinical Pathology*, 2nd ed., 1951, 113
GRANT, R. T., and REEVE, E. B.: *Observations on the General Effects of Injury in Man (With Special Reference to Wound Shock)*, 1951, 116
MOLLISON, P. L., *Blood Transfusion in Clinical Medicine*, 1951, 115
MORISON, J. E.: *Foetal and Neonatal Pathology*, 1951, 118
MUIR'S *Textbook of Pathology*, 6th ed., revised by D. F. Cappell, 1951, 113

BOWIE, J. H., and GOULD, J. C.: Colouring agents for use in disc-antibiotic sensitivity tests, 356
Burns, blood protein-bound carbohydrate after, 194

C

Calcification, coronary arteriosclerosis and, in infancy, 175
Cancer, relationship between serum copper and iron in, 190
Carbohydrate, blood protein-bound, after injury, 194
Carbohydrates in normal and abnormal cerebrospinal fluid, 319
Carbon dioxide requirements of small colony variants of *Staph. aureus*, 354
Carriers of Cooley's anaemia, diagnosis of, 154
Cerebrospinal fluid, carbohydrates in normal and abnormal, 319
— glucose estimation, intrathecal streptomycin and, 199
CHAPLIN, H., JUN., CRAWFORD, H., CUTBUSH, M., and MOLLISON, P. L.: The effects of a phenothiazine derivative (RP.3300) on red cell preservation, 91
Chemistry, clinical, running of standards in, 305
Cholesterol tolerance test in normal individuals, 203
CLARK, A. E., *see* Hutt, M. S. R., Smith, P., Clark, A. E., and Pinniger, J. L.
Coagulohepatogram, construction and clinical significance of, 257
Colouring agents for use in disc-antibiotic sensitivity tests, 356
Containers, screw-capped, aseptic filling of, 290
Control chart, use in clinical chemistry, 305
COOK, G. T., and JEBB, W. H. H.: Starch-fermenting, gelatin-liquefying corynebacteria and their differentiation from *C. diphtheriae gravis*, 161
Cooley's anaemia, diagnosis of carriers of, 154
Copper metabolism in hepatolenticular degeneration, 16
— serum, in patients with neoplastic disease, 190
Coronary arteriosclerosis and calcification in infancy, 175
Corynebacteria, starch-fermenting, gelatin-liquefying, differentiation of, 161
Corynebacterium diphtheriae gravis, differentiation of starch-fermenting, gelatin-liquefying corynebacteria from, 161
Counting chamber, new, for the eosinophil count, 111
CRAWFORD, H., *see* Chaplin, H., jun., Crawford, H., Cutbush, M., and Mollison, P. L.
CROME, L.: The histological changes in hydantoinate gingivitis, 244
Crystalline inclusions, anisotropic non-silicious, in a dysgerminoma, 239
CUTBUSH, M., *see* Chaplin, H., jun., Crawford, H., Cutbush, M., and Mollison, P. L.

D

DAVIDSON, W. M., and JENNISON, R. F.: The relationship between iron storage and anaemia, 281
DAVIES, G., LEVIN, B., and OBERHOLZER, V. G.: The micro estimation of serum iron and iron-binding capacity in normals and in disease, 312
Dextran sulphate as a blood anticoagulant, 366
Diabetes mellitus, liver glycogen in, 42
Differential counts, new type of blood film for, 108
Disc-antibiotic sensitivity tests, 356
DODGE, O. G.: A comparative study of direct matching techniques in blood transfusion, 102
Dye test for toxoplasmosis, standardization of, 350
Dysgerminoma, anisotropic non-silicious crystalline inclusions in, 239

E

EASTHAM, M. D., and KEAY, K. R.: The carbohydrates in normal and abnormal cerebrospinal fluid, 319
Electrophoretic paper strips, apparatus for reading, 294
Embedding media, polyethylene glycols as, 345
Embolism, bone-marrow, 63
Eosinophil count, new counting chamber for the, 111
Epithelial tumours, primary, of the jaw, 225
Erythrocytes, polyagglutinability of, *in vivo*, 266
— trypsinated human, agglutination of, 332
Escherichia coli, pathogenic, media for isolation and differentiation of, 361
Euglena gracilis, vitamin B_{12} assay in body fluids using, 250

F

Faecal fat, rapid method for determining, 288
FAHMY, N. I., and GHALIOUNGUI, P.: The cholesterol tolerance test in normal individuals, 203

Fat, faecal, rapid method for determining, 288
Flame photometer determinations, effect of photo-electric cell temperature on, 286
Flocculation tests, mechanism and interrelationships of, 1
Fluorescin, transfer from *Ps. pyocyanea* to colonies of other bacteria, 359
Folic acid therapy in megaloblastic anaemia of pregnancy, 329

G

Ghalioungui, P., *see* Fahmy, N. I., and Ghalioungui, P.
Gingivitis, hydantoinate, histological changes in, 244
Glucose, cerebrospinal, intrathecal streptomycin and estimation of, 199
Glutathione, blood, determination by amperometric titration, 30
— — nitroprusside method for determination of, 25
Glycogen, liver, in diabetes mellitus, 42
Glycols, polyethylene, as embedding media, 345
Gould, J. C., *see* Bowie, J. H., and Gould, J. C.
Govan, A. D. T., *see* Scott, J. M., and Govan, A. D. T.
Gram-negative bacilli, antibiotic resistance in, 169
Griffiths, L. L.: A direct scanning apparatus for reading electrophoretic paper strips, 294

H

Haemagglutination technique in study of *Trichomonas vaginalis* infections, 275
Haemolysins, ox cell, in human serum, 324
Hall, M., and Ricketts, C. R.: The use of dextran sulphate as a blood anticoagulant in biological research, 366
Heart, angiomyxoma of the auricle, 339
Henig, E., *see* Rappaport, F., and Henig, E.
Henry, R. J., and Segalove, M.: The running of standards in clinical chemistry and the use of the control chart, 305
Hepatolenticular degeneration, amino-aciduria and copper metabolism in, 16
Hewer, T. F.: Primary epithelial tumours of the jaw, 225
Histochemical work, polyethylene glycols as embedding media in, 345
Hübener–Thomsen–Friedenreich phenomenon, 266
Hurst, L., and Lowbury, E. J. L.: Transfer of fluorescin from *Ps. pyocyanea* to colonies of other bacteria, 359
Hutt, M. S. R., Smith, P., Clark, A. E., and Pinniger, J. L.: The value of rib biopsy in the study of marrow disorders, 246
Hydantoinate gingivitis, histological changes in, 244
Hypertension, pulmonary, necrotizing pulmonary arteriopathy associated with, 36

I

Infancy, coronary arteriosclerosis and calcification in, 175
Injury, blood protein-bound carbohydrate after, 194
Intrathecal streptomycin and cerebrospinal glucose estimation, 199
Iron, serum, influence of age upon, 10
— — in patients with neoplastic disease, 190
— — micro-estimation of, 312
— storage and anaemia, 281
Iron-binding capacity of serum, unsaturated, determination of, 271
— — — — — micro-estimation of, 312

J

Jaw, epithelial tumours of, 225
Jebb, W. H. H., *see* Cook, G. T., and Jebb, W. H. H.
Jennison, R. F., *see* Davidson, W. M., and Jennison, R. F.
Johnstone, J. M.: True uric acid values, 317

K

Keay, K. R., *see* Eastham, M. D., and Keay, K. R.
Keyser, J. W.: Blood serum protein-bound carbohydrate after injury, 194
— *see* Spillane, J. D., Keyser, J. W., and Parker, R. A.

L

Lajtha, L. G.: Culture of human bone marrow *in vitro*; the reversibility between normoblastic and megaloblastic series of cells, 67
Lecutier, M. A.: A case of the Schön'ein–Henoch syndrome with myocardial necrosis, 336
Levin, B., *see* Davies, G., Levin, B., and Oberholzer, V. G.
Leyton, G. B.: Ox cell haemolysins in human serum, 324
Linsell, W. D.: The antibiotic sensitivity of pathogenic staphylococci, 165
Liver, glycogen in diabetes mellitus, 42
Lowbury, E. J. L., *see* Hurst, L., and Lowbury, E. J. L.
Lunnon, J. B., *see* Maclagan, N. F., Martin, N. H., and Lunnon, J. B.

M

McEntegart, M. G.: The application of a haemagglutination technique to the study of *Trichomonas vaginalis* infections, 275
Maclagan, N. F., Martin, N. H., and Lunnon, J. B.: The mechanism and interrelationships of the flocculation tests, 1
Manners, T.: A new counting chamber for the eosinophil count, 111
Marrow disorders, value of rib biopsy in study of, 246
Martin, N. H., *see* Maclagan, N. F., Martin, N. H., and Lunnon, J. B.
Media for isolation and differentiation of pathogenic *Esch. coli*, 361
Megakaryocytes in Paget's disease, 86
Megaloblastic anaemia of pregnancy, extensive myeloid response during folic acid therapy in, 329
— anaemias, vitamin B_{12} concentrations of serum and urine in, 129
— and normoblastic series of cells, reversibility between, 67
Micro-estimation of serum iron and iron-binding capacity, 312
Micro-method for blood salicylate estimations, 208
Mollin, D. L., and Ross, G. I. M.: The vitamin B_{12} concentrations of serum and urine of normals and of patients with megaloblastic anaemias and other diseases, 129
Mollison, P. L., *see* Chaplin, H., jun., Crawford, H., Cutbush, M., and Mollison, P. L.
Mooney, F. S.: The diagnosis of carriers of Cooley's anaemia, 154
Moss, D. G.: A micro-method for blood salicylate estimations, 208
Myeloid response during folic acid therapy, 329
Myocardial and renal failure, primary amyloidosis with, 54
— necrosis, Schönlein–Henoch syndrome with, 336

N

Necrotizing pulmonary arteriopathy associated with pulmonary hypertension, 36
Needle, combined sternal biopsy and aspiration, 112
Neoplastic disease, relationship between serum copper and iron in, 190
Nephron nephrosis, lower, after transurethral resection of prostate, 234
Nephrosis, lower nephron, after transurethral resection of prostate, 234
Neufeld, O. E.: A rapid method for determining faecal fat, 288
Nitroprusside method for the determination of blood glutathione, 25
Normoblastic and megaloblastic series of cells, reversibility between, 67

O

Oberholzer, V. G., *see* Davies, G., Levin, B., and Oberholzer, V. G.
O'Hagan, J. E.: A simple method for the aseptic filling of screw-capped containers, 290
Orthotolidine hydrochloride test for blood in urine, 212
Osteitis deformans, bone marrow in, 86
Ostrom, B. W., *see* Spaet, T. H., and Ostrom, B. W.
Ova of *Ascaris lumbricoides*, fragility of, 363
Ox cell haemolysins in human serum, 324

P

Paget's disease, bone marrow in, 86
Panagglutinin, active against trypsinated human erythrocytes, 332
Parker, R. A., *see* Spillane, J. D., Keyser, J. W., and Parker, R. A.
Phenothiazine derivative (RP.3300), effects on red cell preservation, 91
Photo-electric cell temperature, effect on flame photometer determinations, 286
Photometer determinations, flame, effect of photo-electric cell temperature on, 286
Pinniger, J. L., *see* Hutt, M. S. R., Smith, R., Clark, A. E., and Pinniger, J. L.
Pirrie, R.: The influence of age upon serum iron in normal subjects, 10
— Serum copper and its relationship to serum iron in patients with neoplastic disease, 190
Pizzolato, P., and Raider, L.: The bone marrow in Paget's disease (osteitis deformans) with special emphasis on the megakaryocytes, 86
Polyagglutinability of erythrocytes *in vivo*, 266
Polyethylene glyco's as embedding media, 345
Potts, R. E.: Anisotropic non-silicious crystalline inclusions in a dysgerminoma of tuberculoid appearance, 239
Pregnancy, anaemia of, the bone marrow in, 145
— megaloblastic anaemia of, folic acid therapy in, 329
Prostate, transurethral resection of, lower nephron nephrosis after, 234
Pseudomonas pyocyanea, transfer of fluorescin from, 359
Pulmonary arteriopathy, necrotizing, associated with pulmonary hypertension, 36
— hypertension, necrotizing, pulmonary arteriopathy associated with, 36

R

RAEBURN, C.: The histogenesis of four cases of angiomyxoma of the auricle, 339
RAIDER, L., *see* Pizzolato, P., and Raider, L.
RAMALINGASWAMI, V., *see* Sriramachari, S., and Ramalingaswami, V.
RAPPAPORT, F., and HENIG, E.: Media for the isolation and differentration of pathogenic *Esch. coli* (serotypes O 111 and O 55), 361
Red cell preservation, effects of a phenothiazine derivative (RP. 3300) on, 91
REEPMAKER, J.: The relation between polyagglutinability of erythrocytes *in vivo* and the Hübener–Thomsen–Friedenreich phenomenon, 266
Renal and myocardial failure, primary amyloidosis with, 54
Reticulosarcoma of thyroid gland, diagnosis of, 183
Rib biopsy in the study of marrow disorders, 246
RICKETTS, C. R., *see* Hall, M., and Ricketts, C. R.
RIEBEN, W.: The construction and c'inical significance of the coagulohepatogram, 257
RITCHIE, G. M.: Extensive myeloid response during folic acid therapy in megaloblastic anaemia of pregnancy, 329
ROGERS, K. B.: The difficulty caused by the fragility of the ova of *Ascaris lumbricoides*, 363
ROSS, G. I. M.: Vitamin B_{12} assay in body fluids using *Euglena gracilis*, 250
— *see* Mollin, D. L., and Ross, G. I. M.
RP.3300, effects on red cell preservation, 91
Running of standards in clinical chemistry, 305

S

Sa'icylate estimations, micro-method for blood, 208
Scanning apparatus for reading electrophoretic paper strips, 294
Scholarships in aid of scientific research, 364
Schönlein–Henoch syndrome with myocardial necrosis, 336
SCOTT, G. B. D.: The diagnosis of reticulosarcoma of the thyroid g'and, 183
SCOTT, J. M., and GOVAN, A. D. T.: The bone-marrow in the anaemia of pregnancy, 145
Screw-capped containers, aseptic filling of, 290
SEGALOVE, M., *see* Henry, R. J., and Segalove, M.
Serum, copper and iron in patients with neoplastic disease, 190
— iron and iron-binding capacity in normals and in disease, 312
— — in normal subjects, influence of age upon, 10
— ox cell haemolysins in human, 324
— panagglutinin active against trypsinated human erythrocytes, 332
— unsaturated iron-binding capacity of, 271
— vitamin B_{12} concentrations of, in megaloblastic anaemias, 129
SHERRIS, J. C.: Two small colony variants of *Staph. aureus* iso'ated in pure culture from closed infected lesions and their carbon dioxide requirements, 354
SLADDEN, R. A.: Coronary arteriosclerosis and calcification in infancy, 175
SMITH, M. J. H.: Intrathecal streptomycin and cerebrospinal glucose estimation, 199
SMITH, P., *see* Hutt, M. S. R., Smith, P., Clark, A. E., and Pinniger, J. L.
SPAET, T. H., and OSTROM, B. W.: Studies on the normal serum panagglutinin active against trypsinated human erythrocytes. Part I: The mechanism of agg'utination reversal, 332
SPILLANE, J. D., KEYSER, J. W., and PARKER, R. A.: Amino-aciduria and copper metabolism in hepatolenticular degeneration, 16
SRIRAMACHARI, S., and RAMALINGASWAMI, V.: Polyethylene glycols as embedding media for histochemical work, 345
Standards, running, in clinical chemistry, 305
Staphylococci, antibiotic sensitivity of pathogenic, 165
Staphylococcus aureus in pure culture from closed infective lesions, 354
— — two small colony variants of, 354
Starch-fermenting, gelatin-liquefying corynebacteria, differentiation of, 161
Sternal biopsy and aspiration needle, combined, 112
Streptomycin and cerebrospinal fluid estimation, 199
SWINNEY, J., and TOMLINSON, B. E.: Lower nephron nephrosis after transurethral resection of the prostate, 234
SYMMERS, W. ST. C.: Necrotizing pulmonary arteriopathy associated with pulmonary hypertension, 36

T

Thalassaemia, studies on the pathogenesis of, 140
THOMPSON, B. A.: Combined sternal biopsy and aspiration needle, 112
— Device for sampling blood bottles, 365
THOMPSON, R. H. S., and WATSON, D.: The nitroprusside method for the determination of blood glutathione, 25
THOMSON, E. F.: The incidence of antibiotic resistance in Gram-negative bacilli in a general hospital, 169
Thyroid g'and, reticulosarcoma, diagnosis of, 183
TIERNEY, R. B. H.: Bone marrow embolism, 63
TOLENTINO, P., *see* Astaldi, G., and Tolentino, P.
TOMLINSON, B. E., *see* Swinney, J., and Tomlinson, B. E.
Toxoplasmosis, standardization of dye test for, 350
Transurethral resection of prostate, lower nephron nephrosis after, 234
Trichomonas vaginalis infections, haemagglutination technique in study of, 275
Trypsinated human erythrocytes, agglutination of, 332
Tumours, primary epithelial, of jaw, 225

U

Uric acid values, true, 317
Urine, orthotolidine hydrochloride test for blood in, 212
— vitamin B_{12} concentrations of, in megaloblastic anaemias, 129

V

VALLANCE-OWEN, J.: Liver glycogen in diabetes mellitus, 42
VENTURA, S.: Determination of the unsaturated iron-binding capacity of serum, 271
Vitamin B_{12} assay in body fluids using *Eug'ena gracilis*, 250
— — concentrations of serum and urine of patients with megaloblastic anaemias, 129

W

WATSON, D., *see* Bidmead, D. S., and Watson, D.
— *see* Thompson, R. H. S., and Watson, D.
WILLIAMS, A. W.: Primary amyloidosis with renal and myocardial failure, 54
WILSON, A. O.: The effect of photo-electric cell temperature on flame photometer determinations, 286
WOODRUFF, M. F. A.: A new type of blood film for differential counts, 108

Z

ZWARENSTEIN, H.: Orthotolidine hydrochloride test for blood in urine, 212

INDEX TO ABSTRACTS

Entries followed by the letter P in brackets (P) refer to the reports of the General Meetings of the Association of Clinical Pathologists.

A

Actinomycosis, penicillin in, 298
Adenomatoid tumours of genital tract, 303
Amaurotic idiocy, pre-juvenile (P), 213
Amino-acid pattern in human foetal and maternal plasma, 124
Amino-acids, plasma, in liver and renal disease (P), 217
Amoebiasis, intestinal, chronic involvement of liver in, 223
Anaemia, acquired haemolytic, use of trypsin-modified erythrocytes as test cells in, 302
— aplastic, 126
— — fatal case following chloramphenicol therapy, 123
— atypical congenital haemolytic (P), 214
— Cooley's, foetal haemoglobin in, 302
— Mediterranean, inheritance of (P), 372
— megaloblastic, of pregnancy and puerperium, 301
— — vitamin B_{12} in serum and urine of patients with (P), 214
— of thermal injury, 125, 126
Anaemia, pernicious, development of concept of (P), 372
Antibiotics, difficulties in use of (P), 367
— effect on *Ps. aeruginosa* and *Proteus vulgaris*, 299
— newer, bacteriologic studies of, 119
— — in pertussis, 221
— selective distribution in animal organs (P), 216
— steatorrhoea following use of, 299
— treatment of *H. influenzae* meningitis with, 121
Anti-haemophilic potency, testing of plasma and its fractions for (P), 368
Antimycotic chemotherapy, 299
Antithrombin titer in cystic fibrosis of pancreas, 301
Aplastic anaemia, 126
— — fatal case following chloramphenicol therapy, 123
Atherosclerosis, coronary, relationship of degree with age, 128
Aureomycin in surgical infections, 298
— use in children, 121

B

Bacteria, pathogenic, survival on cotton-wool swabs, 373
Bacteriostatic activity, electrolytes and *in vitro* (P), 216
Bacterium coli, specific serological types of, in children (P), 216
Barbiturate levels in cases of intoxication (P), 219
Blood and urine diastase during acute pancreatitis, 223
— constituents, normal values for (P), 370
— count, physiological variations in (P), 372
— in urine, chemical tests for, 299
— occult, in faeces, detection of, 300
— of cyclotron workers, unusual finding in, 302
— pyruvic acid estimations during glucose tolerance test, 223
— transfusion, by siliconelined syringe in haemorrhagic thrombocytopathia, 223
— — compatibility tests preceding (P), 214
— — during 1951 in hospital group of 700 beds (P), 215
— — errors in cross-matching test (P), 215
Body water, total, N-acetyl 4-aminoantipyrine in measurement of, 300
Bone marrow, aspiration, neoplastic cells in, 374
— — disorders, rib biopsy in (P), 214
— — in hyperthyroidism and hypothyroidism, 302
Brucellosis, new method for treatment of, 123
Burns, liver function in, 126
— pigment excretion in patients with, 125
— renal function studies in, 373
Burying, disposal of the body by (P), 220

C

Calciferol, action in lupus vulgaris (P), 371
Cancer as sequel to war wounds, 374
— primary, of liver, 304
Carcinoma, hepatic, in haemochromatosis, 304
Cardiac glycogen storage disease, familial, 304
Chemotherapy, antimycotic, 299
Chloramphenicol, fatal aplastic anaemia following, 123
— in bacillary urinary infections, 221
— in dysentery due to sulfonamide resistant *Shigella sonnei*, 122
— in *H. influenzae* meningitis, 121
— in typhoid, 122
— in typhoid or paratyphoid fever in infants, 122
Chloromycetin, *see* Chloramphenicol
Cirrhosis of liver, water retention associated with, 300
Coagulation defect in tromexan therapy (P), 369
— — from Rh isosensitization, 224
— time, normal, haemophilia associated with, 127
Compatibility tests, preceding transfusion (P), 214
Complement, nature of (P), 218
— role in Donath–Landsteiner reaction, 127
Cooley's anaemia, presence of foetal haemoglobin in, 302
Coronary atherosclerosis, relationship of degree with age, 128
Cortisone therapy, effect on histology of rheumatoid arthritic joints (P), 219
Cross-matching test, errors in (P), 215
Cryoglobulinaemia in multiple myelomatosis, 374
Cyclotron workers, unusual finding in blood of, 302
Cystadenoma lymphomatosum, papillary, of the parotid gland, 224
Cystic fibrosis of pancreas, antithrombin titer in, 301
Cytochemical methods in diagnostic haematology (P), 369

D

Diastase, blood and urine, during transient acute pancreatitis, 223
Diphtheria carriers, treatment with tyrothricin, 120
Donath–Landsteiner reaction, role of complement in, 127
Dudgeon's rapid smear technique, diagnostic value of (P), 213
Dysentery, chloromycetin in, 122
— due to sulphonamide-resistant *Shigella sonnei*, 122

E

Electrolytes and *in vitro* bacteriostatic activity (P), 216
Electrophoresis, strip paper (P), 370
Embryological tumour of retinal anlage involving the skull, 224
Enzyme levels, serum, in perforated peptic ulcer, 222
Eosinophil counting, modification of Pilot's method, 374
Epinephrine, hypersplenic syndromes before and after, 126
Epithelioma, self-healing, of skin, 303
Erythrocytes, trypsin-modified, as test cells in acquired haemolytic anaemia, 302

F

Faeces, detection of occult blood in, 300

G

Gastric arteries, pathology of (P), 367
— tumours, benign polypoid (P), 367
Genital tract, mesotheliomas of, 303
Glucose tolerance test, value of blood pyruvic acid estimations during, 223
Glycogen storage disease, 304
Gout, effects of drugs on plasma uric acid and urinary uric acid in, 373

H

Haematology, cytochemical methods in diagnostic (P), 369
Haematuria, sulphonamide, 120
Haemochromatosis, primary carcinoma of liver in, 304
Haemoglobin, foetal, in Cooley's anaemia, 302
Haemoglobinuria, paroxysmal cold, haemolysis in, 127
Haemolysis in paroxysmal cold haemoglobinuria, 127
Haemolytic anaemia, atypical congenital (P), 214
— disease of newborn, conservative treatment of (P), 372
Haemophilia associated with normal coagulation time, 127
— in the female, 127
Haemophilus influenzae meningitis, chloramphenicol and other antibiotics in, 121
Haemorrhagic thrombocytopathia, blood transfusion in, 223
Hepatitis, chronic amoebic, 223
— homologous serum, apparently caused by human thrombin, 374
Hypersplenic syndromes before and after epinephrine, 126
— — splenic arterial and venous blood studies, 126
Hypertension, primary pulmonary, 128
Hyperthyroidism, bone marrow in, 302
Hypothyroidism, bone marrow in, 302

I

Idiocy, amaurotic, pre-juvenile (P), 213
Inclusion disease, 124
Iron, histologically demonstrable, in sternal marrow biopsies (P), 369
— metabolism in pregnancy, 300
Isoniazid, sensitivity and resistance of *M. tuberculosis* to (P), 371

L

Leukaemia, acute, streptomyces fermentation derivatives in, 302
Leukaemic cells, primitive, differentiation of (P), 368
Lingular biopsy in mitral stenosis (P), 368
Liver, chronic involvement in intestinal amoebiasis, 223
— disease, plasma amino-acids in (P), 217
— function in patients with burns, 126
— in newborn, circulatory changes in (P), 213
— primary cancer of, 304
— primary carcinoma of, in haemochromatosis, 304
Lupus vulgaris, action of calciferol in (P), 371

M

Medicine, modern, the individual and the group in (P), 372
Megaloblastic anaemia of pregnancy and puerperium, 301
— — vitamin B_{12} in serum and urine of patients with (P), 214
Melanomata, visceral, origin of (P), 368
Meningitis, *H. influenzae*, chloramphenicol and other antibiotics in, 121
— terramycin in, 298
Mesothelioma of female genital tract, 303
Mesotheliomas, benign, of genital tract, 303
Mitral stenosis, lingular biopsy in (P), 368
Mycoses, chemotherapy of, 299
Myocobacterium tuberculosis, sensitivity and resistance to isoniazid (P), 371
Myelomatosis, multiple, cryoglobulinaemia in, 374

N

NAAP in measurement of total body water, 300
N-acetyl 4-aminoantipyrine in measurement of total body water, 300
Naevi, cellular, 304
Necropsy findings in three members of a family (P), 220
Neomycin, results of clinical use of, 120
Neoplastic cells in bone marrow aspiration, 374
Nocardamin, a new antibiotic, 221
Nocardia, new antibiotic from species of, 221

P

Pancreas, antithrombin titer in systic fibrosis of, 301
Pancreatitis, transient acute, blood and urine diastase during, 223
Papain-treated red cells in detection of rhesus antibody (P), 372
Paper strip electrophoresis (P), 370
Paratyphoid in infants, chloramphenicol in, 122
Parotid gland, papillary cystadenoma lymphomatosum of, 224
Penicillin in actinomycosis, 298

Peptic ulcer, fatal haemorrhage from (P), 367
— — perforated, raised serum-enzyme levels in, 222
Periarteritis nodosa, renal lesions in, 128
Pertussis, newer antibiotics in, 221
Phenols, plasma, in liver and renal disease (P), 217
Phosphatase reaction, identification of *Staph. pyogenes* by, 222
Phosphorus, radioactive, in treatment of polycythemia vera, 127
Pigment excretion in patients with burns, 125
Pilot's method of eosinophil counting, 374
Plasma amino-acids and phenols in liver and renal disease (P), 217
— human foetal and maternal, amino-acid pattern in, 124
— labile factor, activity in disease, 125
— testing of, for anti-haemophilic potency (P), 368
Pleurodynia, epidemic, aetiology of, 124
— — viruses isolated from outbreak of, 124
Polycythemia vera, treatment with radioactive phosphorus, 127
Polymyxins, oral, in chronic *Shigella* infections, 373
Potassium deficiency, early detection of (P), 369
Pregnancy, iron metabolism in, 300
— megaloblastic anaemia of, 301
Proteus vulgaris, effect of antibiotics on, 299
Pseudomonas aeruginosa, effect of antibiotics on, 299
Pseudomonas pyocyaneus, renal localization in mice (P), 371
Puerperium, megaloblastic anaemia of, 301
Pulmonary hypertension, primary, 128
Pyruvic acid estimations during glucose tolerance test, 223

Q

Q fever in Great Britain, causative agent of, 124

R

Radioactive and metabolic methods of thyroid investigation (P), 218
— phosphorus, in treatment of polycythemia vera, 127
Red cells, papain-treated, in detection of rhesus antibody (P), 372
Renal clearance tests, single injection methods for (P), 218
— disease, plasma amino-acids in (P), 217
— function studies in severely burned patients, 373
— lesions in periarteritis nodosa, 128
— localization of *Ps. pyocyaneus* in mice (P), 371
Resins in treatment of water retention associated with cirrhosis of liver, 300
Retinoblastoma, pigmented, 224
Rhesus antibody, papain-treated red cells in detection of (P), 372
— isosensitization, coagulation defects with intrauterine death from, 224
Rheumatoid arthritic joints, effect of cortisone therapy on (P), 219
Rib biopsy in marrow disorders (P), 214

S

Salivary gland virus infection, generalized, 124
Serum enzyme levels, raised, in perforated peptic ulcer, 222
Shigella infection in children, oral polymyxins in, 373
Shigella sonnei dysentery due to sulphonamide resistant, 122
Sickle-cell trait, inheritance of (P), 372
Skin, self-healing epithelioma of, 303
Skull, embryological tumour of, 224
Spirochaetes, Vincent's, demonstration of, 373
Staphyloccus pyogenes, identification by phosphatase reaction, 222
Steatorrhoea, following use of antibiotics, 299
Sternal marrow biopsies, histologically demonstrable iron in (P), 369
Stomach, benign polypid tumours of (P), 367
Streptomyces, fermentation derivatives in acute leukaemia, 302
Streptomycin, enhancement of action of, 121
— resistance in strains of tubercle bacilli, 221
Sulphonamide haematuria, 120
Swabs, cotton-wool, survival of pathogenic bacteria on, 373

T

Terramycin, clinical observations on, 121
— in meningitis, 298
Test meal, tubeless (P), 370
Tetanus, combined active-passive re-immunization against, 119
Thermal injury, anaemia of, 125, 126
Thrombin, human, epidemic of homologous serum hepatitis apparently caused by, 374
Thrombocytopathia, haemorrhagic, blood transfusion by siliconelined syringe in, 223
Thrombocytopenic purpura, thrombocytopenic factor in blood in, 302
Thyroid investigation, radioactive and metabolic methods of (P), 218
Toxoplasmosis, incidence of north-west England, 123
— transmission of antibody from mother to foetus, 123
Transfusion, compatibility tests preceding (P), 214
Tromexan therapy, coagulation defect in (P), 369
Trypsin-modified erythrocytes as test cells in acquired haemolytic anaemia, 302
Tubeless test meal (P), 370
Tubercle bacilli, cultures from laryngeal swabs and gastric washings, 222
— — streptomycin resistance in strains of, 221
Tuberculosis investigations, risk in routine (P), 371
Typhoid fever, chloramphenicol in, 122
— — in infants, chloramphenicol in, 122
Tyrothricin, diphtheria carriers treated with, 120

U

Uric acid, plasma and urinary, in gout, 373
Urinary infections, bacillary, chloramphenicol in, 221
Urine, chemical tests for blood in, 299
Uterus, mesothelioma of, 303

V

Vincent's spirochaetes, demonstration of, 373
Virus infection, generalized salivary gland, 124
Viruses isolated from outbreak of epidemic pleurodynia, 124
Vitamin B_{12} group of factors, 303
— — in serum and urine of patients with megaloblastic anaemia (P), 214

W

War wounds, cancer as sequel to, 374
Wassermann reactions, biological reactions in (P), 217
Water retention associated with cirrhosis of liver, 300
Wounds, war, cancer as sequel to, 374

AUTHORS OF ARTICLES ABSTRACTED

A

Aagard, K., 120
Angrist, A., 301
Armstrong, C. W. J., 299
Axelrod, A. R., 302
Axelrod, J., 300

B

Baar, H. S., 213
Baglione, L., 120
Baker, T. E., 123
Bangle, R., 304
Barber, M., 222
Barnard, R. D., 302
Barnes, R. W., 221
Barnes, S. W., 302
Basora y DeFillo, J., 121
Beidleman, H., 120
Bene, E., 373
Benjamin, J. W., 301
Benjamin, M., 373
Berger, E. Y., 300
Bergman, R. T., 221
Berman, L., 302
Blades, A. N., 374
Blake, F. G., 121
Bonfils, S., 223
Booher, C. E., 221
Boon, T. H., 126
Bouronclc, B. A., 126, 127
Brack, A., 221
Brodie, B. B., 300
Bruger, M., 300
Buckingham, M., 124
Burkhardt, R., 302

C

Canetti, G., 221
Caplan, H., 299
Cecil, G. W., 374
Charteris, A. A., 303
Clancy, C. F., 120
Clarke, B. E., 224
Cooper, M. L., 302
Crumpler, H. R., 124

D

Dacie, J. V., 214, 368
Damashek, W., 223, 302
Dankner, A., 223
Darmady, E. M., 217
Davson, J., 128
De Bakey, M. E., 373
Deltour, G., 223
Denman, F. R., 373
Dent, C. E., 124
Diamond, L. K., 224
Dingle, J. H., 127
Discombe, G., 299
Doan, C. A., 126
Dockerty, M. B., 303
Douglas, A. S., 369
Drake, W. L., 304
Dry, T. J., 128
Duncan, G. G., 120
Dunning, M. F., 300
Durant, J. A., 217, 370
Dyke, S. C., 372

E

Edwards, J. E., 128
Emery, J. L., 213
Enders, J. F., 124
Enticknap, J. B., 368
Evans, E. I., 125, 126

F

Farinaud, M. E., 122
Farrell, J. B., 221

Favre-Gilly, J., 223
Fine, J., 298
Finn, J. J., 124
Fisch, C., 300
Flindthausen, E., 120
Földvari, F., 298
Foote, J. B., 218
Friou, G. J., 121

G

Gennes, L. de, 223
Gibson, 213
Gilbertson, E., 127
Gilje, A., 222
Gillis, L., 374
Glover, D. M., 224
Goldsmith, K., 372
Gorrill, R. H., 371
Gray, J. D. A., 367

H

Hall, H. E., 121
Hamolsky, M. W., 374
Harkness, J., 217, 370
Harrington, W. J., 302
Hayhoe, F. G. J., 369
Haynes, B. W., 373
Heifetz, C. J., 223
He'mhold, R., 121
Herrell, W. E., 123
Hirsch, I., 223
Hoffman, A., 299
Hoffmann, R. J., 123
Holden, H. S., 220
Hollingsworth, J. W., 302
Holmberg, R. E., 224
Horley, J. F., 372
Horn, R. C., 303
Hoyne, A. L., 298
Hughes, W. H., 371
Humble, J. G., 372
Hunt, A. D., 121
Hutchison, H. E., 369
Hutt, M. S. R., 214

I

Imburg, J., 121
Ingram, M., 302
Innerfield, I., 301
Israëls, M. C. G., 127

J

James, G. W., 125, 126
Jawetz, E., 373
Jordan, W. S., 127

K

Keller, H. M., 122
Kersley, G. D., 373
King, D., 219
King, E. J., 370
Klopper, A., 300
Knox, R., 371
Kuper, S. W. A., 222

L

Lacey, B. W., 216
Landing, B. H., 304
Larner, A. E., 299
Lee, M. J., 303
Lee, S., 374
Lempert, H., 127
Lesses, M. F., 374
Lewis, G. C., 303
Lieberman, D., 373
Lindan, O., 124
Liquori, A. M., 302
Lloyd, O. C., 368
Lopez, J. Chabás, 299
Loud, P. A., 217

M

McCrumb, F. R., 121
MacDonald, A., 123
MacFarlane, J. C. W., 374
Mackenzie, D. H., 218
Maclagan, N. F., 218
Mandel, L., 373
Marie, J., 122
Marks, J., 372
Marland, P., 122
Marrack, J. R., 218
Martin, N. H., 370
Masson, P., 304
Merideth, A., 121
Merliss, R. R., 299
Merskey, C., 127
Miller, J. L., 119
Minnich, V., 302
Mollin, D. L., 214
Mollison, P. L., 214 (2)
Moore, C. V., 302
Moser, R. H., 300
Myers, W. G., 127

N

Naftalin, L., 369
Nickolls, L. C., 219
Nissen, N. I., 120
Nola, F. di, 120

O

Osborn, G. R., 367

P

Parsons, H., 224
Peranio, A., 300
Pickett, R. D., 300
Pillemer, L., 127
Pinniger, J. L., 214
Pitney, W. R., 368
Porosowska, Y., 300
Portes, L., 122
Pratt, J., 223
Price, D. E., 220
Pulvertaft, R. J. V., 372
Purnell, O. J., 125, 126

R

Rees, R. J. W., 121
Reid, D. E., 224
Renz, J., 221
Rice, H. M., 217
Rich, M. L., 123
Richards, H. G. H., 367
Richardson, N., 214
Riff, E. R., 298
Ritterhoff, R. J., 123
Robson, J. M., 121
Roby, C. C., 224
Rohn, R. J., 127
Rosenak, B. D., 300
Rosenthal, M. C., 302
Ross, G. I. M., 214
Rubbo, S. D., 373
Rubinstein, M. A., 374
Rutenberg, A. M., 298
Ryan, M. L., 119

S

Saenz, A., 221
Sandler, R., 223
Schweinburg, F. B., 298
See, G., 122
Selwyn, J. G., 214
Seringe, P., 122
Smelin, A., 374
Smith, D. Robertson, 368
Smith, E. Lester, 303
Smith, M. G., 124
Smith, P., 214
Spencer, H., 128
Spicer, S., 119
Steele, J. M., 300
Stefanini, M., 125
Stewart, C. P., 218
Stewart, G. T., 216
Stoker, M. G. P., 124
Stoll, A., 221
Stratton, F., 215

T

Tashman, S. G., 121
Thompson, G. J., 303
Thompson, R. B., 301
Tovey, G. H., 215

U

Ungar, J., 216
Ungley, C. C., 301

V

Vago, S. C., 373
Vellios, F., 124
Ventura, S., 300

W

Wagner, R. R., 121
Wainwright, J., 128
Walton, J. N., 126
Wapshaw, H., 222
Warren, S., 304
Waugh, J. M., 303
Weiner, A. E., 224
Weller, T. H., 124
West, E. J., 221
Wetherley-Mein, G., 371
White, N. K., 128
Whitlock, C. M., 121
Wilson, D., 219
Wilson, W. M., 221
Wiseman, B. K., 127
Wolgamot, J. R., 120
Wootton, I. D. P., 370
Wright, C. S., 126

Z

Zeitlhofer, J., 304
Zollinger, R. M., 126